Cloning and functional analysis of the genes from entomopathogenic fungi involved in the biosynthesis of eicosatetraenoic acid (ETA)

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ABSTRACT

Very long chain polyunsaturated fatty acids (VLCPUFAs) such as arachidonic acid (ARA, 20:4ω6), eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) have been shown to have many health benefits, some of which include lowering blood pressure, providing protection against cardiovascular diseases and improving brain and eye functions. Entomopathogenic fungi, a group of fungal pathogens able to infect insects, were previously reported to produce substantial amounts of VLCPUFAs, however the genes involved in the biosynthesis of these fatty acids have yet to be identified. This research started with fatty acid analysis of five entomopathagenic fungi, of which *Conidiobolus obscurus* and *Conidiobolus thromboides* were found to produce high levels of VLCPUFAs such as ARA and EPA. Thus, these two fungal species were selected as potential gene sources for the enzymes involved in the biosynthesis of VLCPUFAs. Using degenerate reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of the cDNA ends (RACE) methods; we cloned two full-length putative Δ6 desaturase cDNAs (*CoD6* and *CtD6*) from the two fungi.

Functional expression of *CoD6* in *Saccharomyces cerevisiae* showed it codes for a functional Δ6 desaturase, which can introduce a Δ6 double bond into linoleic acid and α-linolenic acid, respectively. However, expression of *CtD6* in *S. cerevisiae* showed it does not have any Δ6 desaturase activity. Using degenerate RT-PCR and RACE, we also cloned two full-length Δ6 elongase cDNAs (*CoE6* and *CtE6*) from the *C. obscurus and C. thromboides* species. Functional expression of these genes in *S. cerevisiae* showed *CoE6* and *CtE6* code for functional Δ6 elongase. Substrate specificity analysis indicated that both preferentially elongate 18-carbon Δ6 desaturated fatty acids, such as γ-linolenic acid and stearidonic acid. In addition, CtE6 can also elongate 20-carbon VLCPUFAs, such as ARA and EPA. The entire eicosatetraenoic acid (ETA, 20:4ω3) biosynthetic pathway was reconstituted in yeast using four genes, *CoD6* (a Δ6 desaturase) and *CoE6* (a Δ6 elongase) from *Conidiobolus obscurus*, *CpDes12* (a Δ12 desaturase) and *CpDesX* (a ω3 desaturase) from *Claviceps purpurea*. Yeast transformant expressing the four genes produced several new fatty acids. Among them, eicosatetraenoic acid (ETA) accounts for approximately 0.1% of the total fatty acids. Although the level of ETA in the transformant is

low, this represents the first report describing the reconstitution of the entire ETA pathway in yeast without exogenous supplementation of any fatty acids.

Acknowledgements

This dissertation is dedicated to my family but most importantly to my parents (Chung Hsing Tan and Mei Tan) for all their hard work they did to get me this far and their continual support they provided me throughout my life.

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List of Abbrevations

ALA: α-linolenic acid

ARA: Arachidonic acid

CpDes12: *Claviceps purpurea* Δ12 desaturase

CpDesX: *Claviceps purpurea* ω3 desaturase

CoD6: *Conidiobolus obscurus* Δ6 desaturase

CoE6: *Conidiobolus obscurus* Δ6 elongase

CtD6: *Conidiobolus thromboides* Δ6 desaturase

CtE6: *Conidiobolus thromboids* Δ6 elongase

DHA: Docosahexaenoic acid

DGLA: Dihomo-γ-linolenic acid

EPA: Eicosapentaenoic acid

ETA: Eicosatetraenoic acid

GLA: γ-linolenic acid

LA: Linoleic acid

RACE: Rapid amplification of cDNA ends

RT-PCR: Reverse transcriptase polymerase chain reaction

SDA: Stearidonic acid

VLCPUFAs: Very long chain polyunsaturated fatty acids

1.0 Introduction

The *Conidobolus* fungi within entomopathogenic fungi are mainly found to inhabit soil or decaying plant materials in tropical areas, particularly in areas near the equator such as Africa, India and Central America. There are over 21 different species within the genus, some of which have been found to be causative agents in human infections, but the two species *Conidobolus thromboides* and *Conidobolus obscurus* in our study here are known to strictly infect aphids (Scorsetti *et al*., 2007). As these two *Condioblous spp.* are particularly fond of the aphid host, *C. thromboides* and *C. obscurus* have been used as bio-pesticides in controlling the aphid population in various crops such as potato, small grain and cotton (Feng *et al*., 1990; Milner and Soper, 1981; Steinkraus and Tugwell, 1997). In addition, these fungal species have also been reported to be able to accumulate substantial amounts of very long chain polyunsaturated fatty acids (VLCPUFAs) (Tyrrell, 1967).

VLCPUFAs such as ARA, EPA and DHA are essential fatty acids for human health and well-being. Dietary supplementations of these VLCPUFAs have shown to provide protection against many chronic diseases and to enhance performance of eyes and brain. Currently, the main sources of the VLCPUFAs are marine fish and fish oils. However, with the steady declining fish population in oceans, the scientific community is under intense pressure to find new alternative sources for these fatty acids to meet the growing demand.

Eicosatetraenoic acid (ETA, 20:4-8,11,14,17) and dihomo-γ-linolenic acid (DGLA, 20:3-8,11,14) are two novel VCLPUFAs that have recently attracted scientific attention because of their unique chemical properties and biological activities in mediating an immune response and maintaining cellular homeostasis in mammals. Unfortunately, they are not unusually found at substantial amounts in nature. Therefore, there is strong interest in searching for novel desaturases and elongases involved in the biosynthesis of these VLCPUFAs and use of these enzymes in heterologous systems to produce the fatty acids as well as their downstream VLCPUFAs for nutraceutical applications.

1.1 Study rationale

Metabolic engineering of oilseed crops have been viewed as one of the most cost-effective and sustainable alternatives for production of VLCPUFAs. The main objective of this research is to identify and characterize novel Δ6 desaturase and Δ6 elongase genes from entomopathogenic fungi which were previously shown to accumulate VLCPUFAs (Tyrrell, 1967), but from which genes involved in the biosynthesis of these fatty acids have yet to be identified. It was assumed that the genes involved in the biosynthesis of VLCPUFAs could be cloned from the entomopathogenic fungi by using degenerate RT-PCR and RACE, and function of the genes could be characterized by expressing them in yeast *S. cerevisiae*. Ultimately, the genes encoding novel Δ6 desaturase and Δ6 elongase from these fungi could be used for metabolic engineering of yeast and plant to produce VLCPUFAs for nutraceutical applications.

2.0 Literature Review

2.1 Very long chain polyunsaturated fatty acids (VLCPUFAs)

Very long chain polyunsaturated fatty acids (VLCPUFAs) are defined as fatty acids with 20 or more carbons and two or more double bonds. These fatty acids often contain an even-numbered carbon chain with methylene-interupted unsaturated double bonds. Two families of polyunsaturated fatty acids (PUFAs) exist in nature: ω3 and ω6 fatty acids. They are structurally classified by the proximity of the first double bond towards the methyl (ω) end of the fatty acyl chain. Fatty acids of the ω3 family contain a double bond located at the third carbon from the methyl end, while those of the ω6 family contain a double bond located at the sixth carbon from the methyl end. The chemical designation of these fatty acids are commonly abbreviated by listing the number of carbons, the number of double bonds and the location of the first double bond from the methyl terminus. For example, docosahexaenoic acid (DHA) is represented as 22:6ω3, indicating a chain length of 22 carbons with six double bonds and the first unsaturated bond located at the third carbon from the methyl end.

VLCPUFAs in mammals mainly exist as the esterified glycerolipid form (SanGiovanni and Chew, 2005) and have very important physiological functions. Firstly, VLCPUFAs are essential components of the cell membranes of various organs and tissues such as the heart, brain, eyes and nervous system, and help to maintain their structure and function. For instance, docosahexaenoic acid (DHA) has been found to be one of the most abundant ω3 fatty acids in the brain and retina (Kitajka *et al*., 2004; Lee *et al*., 2009; SanGiovanni and Chew, 2005). Secondly, VLCPUFAs are precursors for the synthesis of eicosanoids such as prostaglandins, thromboxanes and leukotrienes, which are hormone – like bioactive compounds that help maintain the cell homeostasis and mediate vascular, neural and immune functions (Anderson and Ma, 2009). Thirdly, VLCPUFAs are ligands of certain trans-acting factors that regulate: (a) cell growth and differentiation; (b) lipid, protein and carbohydrate metabolisms. DHA, EPA and ARA all can affect cellular gene expression through regulation of transcriptional factor activities within the nucleus. Those transcriptional factors with the VLCPUFA-binding domain include peroxisome proliferator-activated receptor (PPAR), retinoid X receptors (RXR), nuclear-factor

kappa B (NFkB) and sterol regulatory element binding proteins (SREBPs) (SanGiovanni and Chew, 2005).

In addition to these three main functions, VLCPUFAs also play an important role in helping mammalians absorb fat-soluble vitamins A, D, E, and K from food.

2.1.1 Dihomo-γ-linolenic acid (DGLA) and eicosatetraenoic acid (ETA)

Dihomo-γ-linolenic acid (DGLA) is a ω6 20-carbon fatty acid with three double bonds located at positions 8, 11 and 14 counting from the carboxylic end the chemical structure is shown Figure 2.1. DGLA is synthesized through the addition of a double bond by Δ6 desaturase into linoleic acid, followed by the chain elongation of a two-carbon unit by Δ6 elongase.

Eicosatetraenoic acid (ETA), the ω3 counterpart of DGLA, is a 20-carbon chain fatty acid with four double bonds located at positions 8, 11, 14 and 17 counting from the carboxylic end the chemical structure is shown Figure 2.2. It is synthesized through the introduction of a double bond by Δ6 desaturase into α-linolenic acid, followed by the chain elongation of a two-carbon unit by Δ6 elongase.

DGLA and ETA are not normally found at substantial amounts in microbes and plants. However, these VLCPUFAs have recently attracted scientific interest because they have unique chemical properties and biological activities. In addition, they can serve as precursors for synthesizing anti-inflammatory eicosanoids, such as series 1 or 3 prostaglandins and thromboxanes, which play a very important role in the mediation of an immune response and cellular homeostasis in mammals.

2.2 Biosynthesis of VLCPUFAs

VLCPUFAs are normally obtained through our diet directly or synthesized from two dietary precursor essential fatty acids (EFAs), α-linolenic acid (ALA) and linoleic acid

Figure 2.1 Chemical structure of dihomo-γ-linolenic acid (DGLA)

Figure 2.2 Chemical structure of eicosatetraenoic acid (ETA)

(LA). Mammals, including human beings, cannot synthesize the two EFAs in our bodies because we lack Δ12 and Δ15 desaturase enzymes required to insert the two double bonds into oleic acid. Thus, we are dependent on the dietary sources of these two EFAs for synthesis of the VLCPUFAs. However, even with the abundant consumption of the two EFAs, the conversion efficiency of the two EFAs to VLCPUFAs in our body is very limited. It has been reported from International Society for the Study of Fatty Acids and Lipids (ISSAFAL) that the conversion efficiency of ALA to DHA is at the order of only 1% in infants, and considerably lower in adults (Anderson and Ma, 2009). Thus, to obtain the optimal health benefits produced by VLCPUFAs, we must consume them either through foods or through dietary supplementation. It is suggested that normal individuals should on average consume 250-500 mg daily of very long chain ω3 fatty acids, whereas individuals shown to have signs of coronary heart disease are recommended to consume at least 1g daily of these VLCPUFAs (Lee *et al.*, 2009).

2.2.1 Common pathways for VLCPUFA biosynthesis

The biosynthesis of VLCPUFAs commonly takes place through a series of alternating desaturation where a front-end double bond is introduced and elongation where the fatty acyl chain is lengthened by a two-carbon unit through the condensation of acyl-CoA and malonyl-CoA. The synthesis of both the ω3 and the ω6 fatty acids utilize the same set of enzymes in their respective pathways.

Figure 2.3 shows the biosynthetic pathway of two families of VLCPUFAs. The biosynthesis begins with Δ9 desaturation of 18C-saturated fatty acid where Δ9 desaturase introduces a Δ9 double bond into stearic acid (18:0) to generate oleic acid (18:1ω9). Oleic acid is then desaturated by Δ12 desaturase to produce linoleic acid (LA; 18:2ω6). Linoleic acid can be used as a substrate for the ω6 fatty acid biosynthesis pathway. Concurrently, Δ15 desaturase can introduce a double bond into LA to generate α-linoleic acid (ALA; 18:3ω3), which can then be used as the substrate for the ω3 fatty acid biosynthesis pathway (Leonard *et al*., 2004).

Next, Δ6 desaturase introduces a double bond at the six-carbon position from the carboxylic end of LA and ALA to generate γ-linolenic acid (GLA; 18:3ω6) and stearidonic acid (SDA; 18:4ω3),

respectively. These products can then be elongated with a two-carbon unit by Δ6 elongase to form dihomo-γ-linolenic acid (DGLA; 20:3ω6) and eicosatetraenoic acid (ETA; 20:4ω3), respectively. These products can again be desaturated by Δ5 desaturase, which introduces a Δ5 double bond to the substrates to generate arachidonic acid (ARA; 20:4ω6) and eicosapentaenoic acid (EPA; 20:5ω3). ARA can continue going down the ω6 pathway to generate its final product ω6 docosapentaenoic acid by Δ5 elongation and Δ4 desaturation steps. In addition, ARA can also be desaturated by a ω3 desaturase which introduces a carbon double bond at the 3rd carbon from the methyl end to form EPA, which can continue going down the ω3 pathway through a Δ5 elongation and Δ4 desaturation to form docosahexaenoic acid (DHA; 20:6ω3) (Leonard *et al*., 2004).

The biosynthesis of DHA had been somewhat of a controversy in 1990s. The major debate was centered on whether there existed a Δ4 desaturation step in the VLCPUFA-biosynthetic pathway. The controversy about its existence was cleared when the first Δ4 desaturase was cloned and functionally characterized from *Thraustochytriidae* (Qiu, 2003). However, no information is available to date indicating the presence of a Δ4 desaturase in mammalian cells. To the contrary, evidence tends to point to a Δ4 -independent pathway for DHA biosynthesis in mammals, known as the Sprecher pathway (Luthria *et al*., 1996; Voss *et al*., 1991). In this pathway, the Δ5 desaturated products, EPA and ARA, undergo two sequential elongations, followed by a Δ6 desaturation and finally a retro-conversion process in the peroxisome where the fatty acid becomes shortened by a two carbon unit to yield DHA and DPA (Sprecher, 2000).

2.2.2 Alternative pathways for VLCPUFA biosynthesis

Δ9 elongation/Δ8 desaturation pathway

As described above, the common pathway for the synthesis of VLCPUFAs begins with the Δ6 desaturation of LA and ALA, followed by two-carbon elongation by Δ6 elongase. Then, several sequential desaturation and elongations take place to form the VLCPUFAs

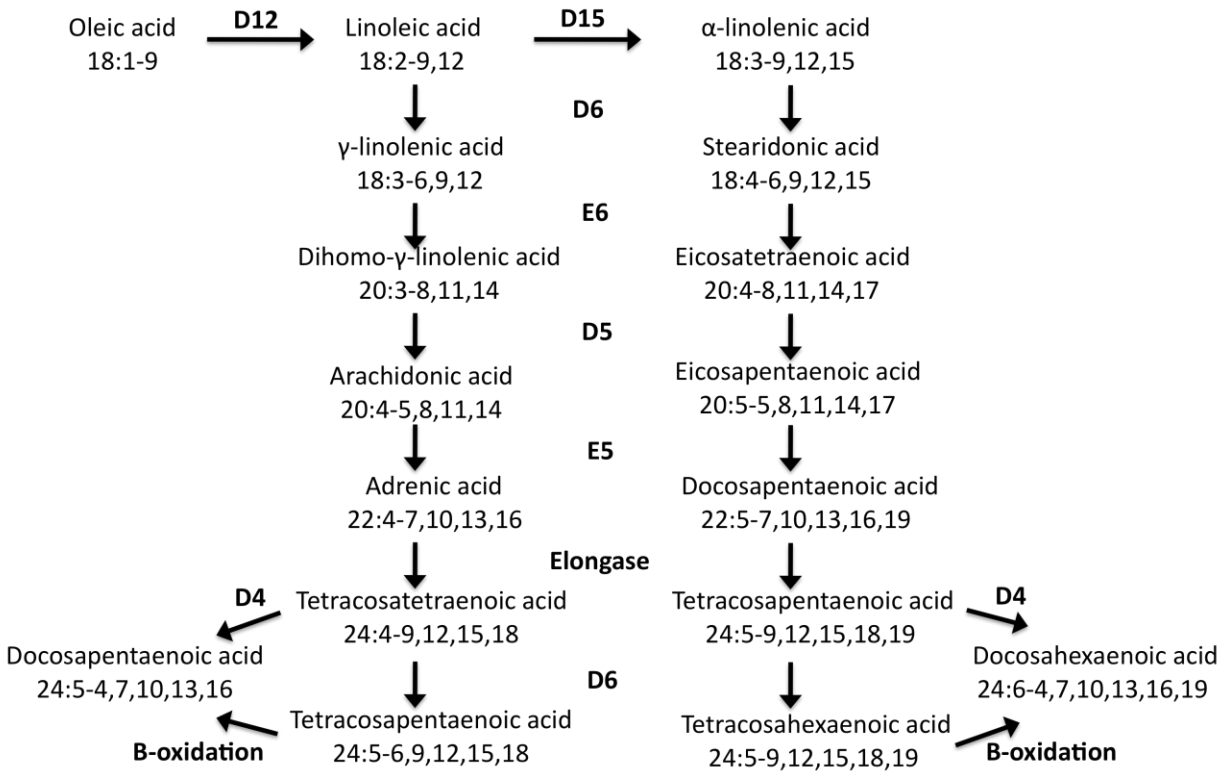


Figure 2.3 The biosynthesis of ω6 and ω3 VLCPUFAs.

such as EPA, ARA and DHA. However, an alternative Δ9 elongation/Δ8 desaturation pathway for VLCPUFAs exits in a few microbial species where LA and ALA are initially elongated to eicosadienoic acid and eicosatrienoic acid, respectively, by Δ9 elongase, which are then desaturated by a Δ8 desaturase to DGLA and ETA (Wallis and Browse, 1999) (Figure 2.4). These products can continue with the latter part of the common VLCPUFA pathway to produce the other VLCPUFAs (Figure 2.3). This alternative pathway for the biosynthesis of VLCPUFAs has been observed in algal *Tetrahymena pyriformis* (Koroly and Conner, 1976), soil amoebae *Acanthamoeba sp.*(Lees and Korn, 1966) and protist *Euglena gracilis* (Wallis and Browse, 1999) where it is the dominant pathway for the synthesis of VLCPUFAs .

Even though the primary focus of recent research is situated around the common aerobic pathway, it is noteworthy that there exists an alternative anaerobic pathway known as the poly-ketide synthase pathway (PKS), which is predominantly found in bacteria and lower eukaryotes. This pathway differs from the alternating desaturation and elongation pathway in that it does not require aerobic desaturation steps to introduce double bonds into the existing fatty acyl chain. Instead, double bonds are introduced through a similar process of fatty acid synthesis carried out by the fatty acid synthase complex where a series of isomerations are used to generate the double bonds (Ferdinandusse *et al*., 2001). This alternative pathway for the synthesis of DHA was observed in *Schizochytrium*, a family member of the *Thraustochyrid* (Metz *et al*., 2001).

2.3 Microbial sources of VLCPUFAs

Currently, fish oils are the major commercial source of the VLCPUFAs for human dietary supplementation. The quality of fish oil, however, depends on fish species, growing season/climate, geographical location of the catching sites and the quality of food consumed by the fish. There have been reported cases of fish oils contaminated with lipid soluble environmental pollutants (Wen and Chen, 2003). Therefore, alternative sources of the VLCPUFAs that are safe, cost-effective and sustainable are being sought as replacements of fish oil (Guschina and Harwood, 2006).

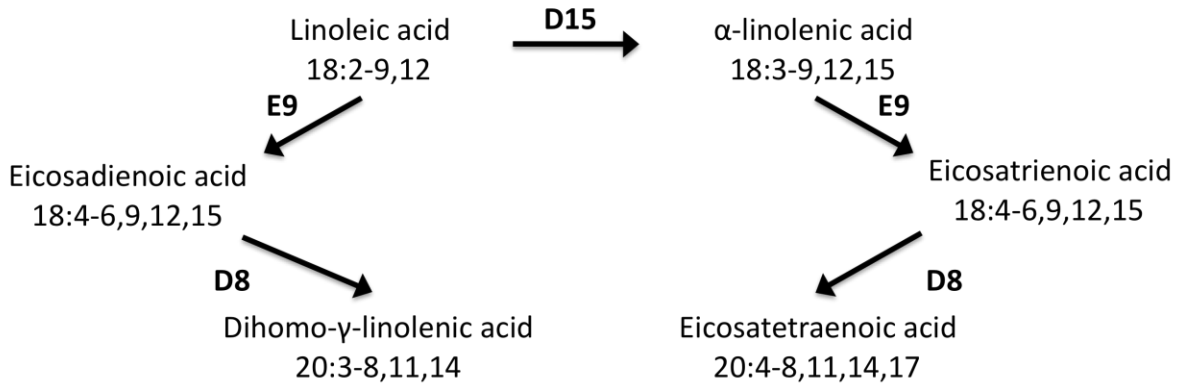


Figure 2.4 The alternative Δ9 elongation/Δ8 desaturation pathway observed in *Euglena sp.*

Algal and fungal oils have recently been explored as new sources for VLCPUFAs; however, the low yields and high cost of oil extractions from the microbes have limited the wide use of these sources. Reconstitution of the VLCPUFA biosynthetic pathway in an oil seed crop to produce VLCPUFAs is considered an attractive alternative, as it appears to be economical and sustainable. By introducing genes involved in the biosynthesis of VLCPUFAs from VLCPUFA-producing microorganisms into oilseed crops, we are able to modify the seed oil composition so that VLCPUFAs could be effectively produced. Over the years, algae and fungi have been the primary gene sources for VLCPUFA production in transgenic plants, as the use of animal genes may cause consumer resistance and ultimately deter the investment required for the development of this alternative VLCPUFA source.

2.3.1 Algae

Eukaryotic algae are very diverse groups of photosynthetic organisms, which inhabit a wide range of ecosystems from the Antarctic to the tropical oceans. As they are the primary producers of the biomass in oceans, microalgae have drawn great scientific attention in recent years. However, studies on the algal lipid biochemistry have recently shifted from a few model organisms to large number of unusual algae that can produce VLCPUFAs. The major drive for this is to discover genes that code for desaturases and elongases involved in the biosynthesis of VLCPUFAs such as ARA, EPA and DHA (Guschina and Harwood, 2006). For example, *Isochyrsis galbana*, *Euglena gracilis* and *Crypthecodinium cohnii* have been well studied recently because of their ability to accumulate large amounts of DHA. These algal species live in a wide range of habitats, which are subjected to multiple forms of stress including temperature, nutrient availability and salt concentrations, thus, they have developed specific mechanisms to adapt to their environments. For instance, when growing under the cold condition, algae can increase the VLCPUFA-biosynthetic activity, which helps maintain membrane fluidity at the lower temperature. There is evidence revealing the direct correlation between the production of Δ6 desaturase mRNA and the temperature shift from 30°C – 22°C in *Mucor circinelloides* (Michinaka *et al*., 2003).

Many genes involved in the biosynthesis of VLCPUFAs have been cloned from algae. Two cDNA clones encoding a Δ12 and ω3 fatty acid desaturase have been isolated from *Chlorella*

vulgaris based on sequence homology to plant Δ12 and ω3 desaturases, which desaturate oleate to linoleate and linoleate to α-linolenate, respectively. Two desaturases (Δ5 and Δ 6 desaturases) were recently identified from marine diatom *Phaeodactylum tricornutum* involved in the EPA biosynthesis (Domergue *et al*., 2002). The first Δ4 desaturase was cloned from *Thraustochytrium spp.*, which proved the existence of the functional Δ4 pathway (Qiu *et al*., 2001). The first non-animal "acyl-CoA" Δ6 desaturase capable of introducing a carbon double bond at the 6th position of the acyl-CoA form of linoleic acid and α-linolenic acid was identified from *Osteococcus tauri* (Domergue *et al*., 2005). The first Δ9 elongase enzyme was cloned from *Isochyrsis galbana*, which operates in the alternative Δ9 elongase / Δ8 desaturase pathway (Wallis and Browse, 1999).

2.3.2 Fungi

Fungi, particularly in the phylum of Zygomycota, have been shown to accumulate large amounts of polyunsaturated fatty acids. Many species of Zygomycota are able to convert excess sugars or other carbon sources into lipids, primarily triacylglycerides. If the carbon is already in the form of fatty acid lipids, they are often taken up and channeled directly into the storage reserves without major chemical modifications. Fungi are given the term as "oleaginous" if they are able to accumulate more than 20% of their dry weight as lipids. Many species of Zygomycota are oleaginous; some of the most well known species are within the genus of *Mortierella spp.* and the *Mucor spp.* (Weber and Tribe, 2003).

One of the typical microscopic features of the hyphae of many Zygomycota is the coarse appearance of their cytoplasm, which is due to the presence of large lipid storage droplets. Lipids are the most important space-saving way to store energy, which is the major reason why they are deposited in the spores of many fungi. These lipid reserves fulfill important functions in starting the metabolism of dormant spores and in fuelling the spore germination (Weber and Tribe, 2003).

Some oleaginous fungi produce TAGs enriched with VLCPUFAs with pharmaceutical/nutraceutical potentials. Within the genus of *Mortierella, M. alpina* is a well-known producer of arachidonic acid, while *M. hyaline* produces eicosapentaenoic acid as well as

arachidonic acid. *Mucor rouxii* produces large amounts of γ-linolenic acid. These fungi have considerable biotechnological potentials as alternative sources of VLCPUFAs or as gene sources for heterologous production of VLCPUFAs in plants.

To date many different VLCPUFA genes have been cloned from fungi. Some of the most well known include Δ6 desaturase from the Zygomycetes *M. rouxii* (Laoteng *et al*., 2000) and Δ5 desaturase from *M. alpina* (Michaelson *et al*., 1998a), ω3 desaturase from *Clavicep purpurea* (Meesapyodsuk and Qiu, 2008). In addition, the very first Δ6 elongases was cloned from *M. alpina* (Parker-Barnes *et al*., 2000).

2.4 Entomopathogenic Fungi

Entomopathogenic fungi (which translates to insect destroyer in Greek) are known to cause decimating epizootics in insects. The insect hosts of these fungi span over 32 different families. Some entomopathogenic fungi have a wide range of hosts, while others are restricted to only single specie or a closely related group of species. Entomophthorale fungi are regarded as one of the most important pathogens of aphids with over 10 different species infecting this insect (Scorsetti *et al.*, 2007; Tyrrell, 1966).

Entomopathogenic fungi are a large order of fungi traditionally classified in Zygomycetes. Most species of entomopathogenic fungi are known to be insect pathogens, while a few can attack nematodes and mites, and some are even found as free-living saphrotrophs in soil or dung. This particular order of fungi is characterized by the presence of a unique sporangium that has been reduced to function as a single conidium. The conidium is capable of producing asexual ballistic spores that are forcibly discharged once the fungus has reached maturity. The mycelia of the fungi consist of coenocytic (multi-nucleate cell) hyphae, in which transverse walls occur infrequently. The hyphae walls are thick and mainly composed of chitin that is colorless or tinted in a shade of brown color (Tanada and Kaya, 1993).

2.4.1 General life cycle of entomopathogenic fungi

Shown below in Figure 2.5 is a generalized life cycle of entomopathogenic fungi. Initially the conidiophore in many species forcibly discharges the conidium, which is covered with a sticky

mucilaginous substance. When this spore lands on a moist substrate other than a host, it will start the formation of the secondary conidiophore and its secondary conidium, which may further develop to produce a tertiary condiophore and conidium, this process may continue until the protoplasm is depleted (Tanada and Kaya, 1993).
Secondary and tertiary conidia may become resting spores. A special type of secondary conidium called a capilliconidium may also form, which is produced on the slender capillary tube (conidiophore) developed on the primary or secondary conidium. Found on the surface of the capilliconidium is a sticky substance which aids in the adhering to objects it may come in contact with, while in other entomopathogenic fungi the secondary conidia and capilliconidia are the primary infective forms.

Sexual reproduction of entomopathogenic fungi occur by the fusion of the mycelium fragments or the hyphal bodies to form a thick-wall zygospore or resting spore. While asexual reproduction can take place by various means: (1) hyphal breaking up into hyphal bodies, (2) primary uni- or multinucleate conidia (primary spores) formed singly at the apex of simple branched conidiophores, (3) secondary conidia (secondary spores) that are produced from the germination of the conidia, and (4) the formation of tertiary conidia from the secondary conidia (Tanada and Kaya, 1993).

Conidobolus genus within entomopathogenic fungi are mainly found to inhabit soil or decaying plant materials in tropical areas, particularly in areas near the equator such as Africa, India and Central America. There are over 21 different species within the genus, some of which have been found to be causative agents in human infections, but the two species in our study (*Conidobolus thromboides* and *Conidobolus obscurus*) are known to strictly infect insects. They were obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (Scorsetti et *al.*, 2007).

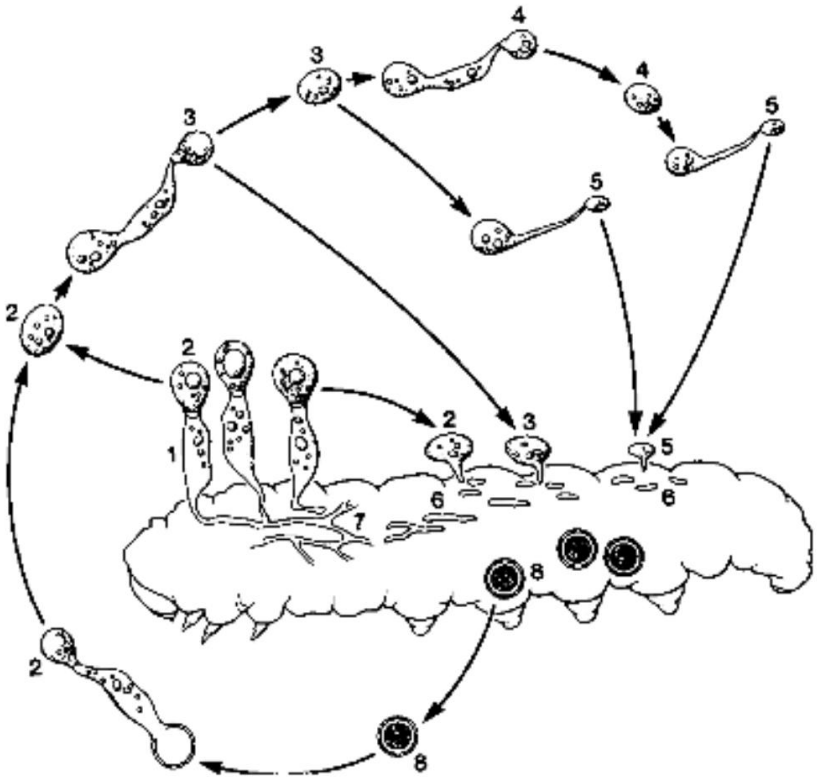


Figure 2.5 The generalized life cycle of entomopathogenic fungi (Reproduced from from Tanada and Kaya, 1993). Under the right conditions the fungus will generate a conidiophore (1), which forcibility ejects a primary conidium (2); this can further develop in a secondary conidium (3) and a tertiary condium (4). These secondary and tertiary structures can land and infect the host promoting the formation of hyphal bodies (6) and mycelia and stroma (7) or the formation of resting spores (8) under conditions where the fungus is unable to find a suitable host. The secondary condium will germinate into a special conidiophore known as the capillicondium (5), which helps it to adhere and stick to other surfaces.

Conidiobolus thromboides and *C. obscurus* have been used as a natural insecticide. They are particularly fond of the aphid host and can be an effective means in controlling the aphid population in various crops such as potato, small grain and cotton (Soper, 1981; Feng *et al.*, 1990; Steinkraus *et al.*, 1997). Besides, these fungi have also been shown to be able to accumulate substantial amounts of VLCPUFAs (Tyrrell, 1967), although their biosynthetic mechanisms in the fungi are not known.

2.5 Cloning and functional characterization of Δ6 desaturases and Δ6 elongases involved in the biosynthesis of VLCPUFAs

2.5.1 Desaturases

Desaturases are ubquitious enzymes found throughout the protist, animal and plant kingdoms, with scattered distribution among eubacteria. They are present in these systems in one of two forms: either as a soluble enzyme or as a membrane bound enzyme, but in plants, both forms are present. Desaturase enzymes fall into the group of di-iron oxo enzymes. They are responsible for catalyzing the reaction of removing two hydrogen atoms and creating a carbon double bond in a fatty acyl chain. There are many types of desaturases with different regioselectivity found throughout the fatty acid modification pathway for creating double bonds at different locations in different fatty acyl chains, and ultimately creating different types of new fatty acids. Shown below in Equation 2.1 is the general reaction that occurs during the desaturation process (Parker-Banes *et al.*, 2000).

$$R_1\text{-}CH_2\text{-}CH_2\text{-}(CH_2)_n\text{-}COOH + O_2 + 2e^- + 2H^+ \text{--}> R_1\text{-}CH{=}CH\text{-}(CH_2)_n\text{-}COOH + 2H_20$$

Equation 2.1 Chemical equation representing the fatty acid desaturation reaction. Fatty acid desaturase, in the presence of molecular oxygen and two reducing equivalents (NADPH), introduces a carbon double bond into a fatty acid with the co-product of water.

Soluble desaturases, also known as acyl-carrier protein (ACP) desaturases represented by the steroyl-ACP desaturase found in the cholorplast of plants only, are able to catalyze the

desaturation of substrates that contain the ACP moiety attached to the fatty acyl chain. For example, the desaturation of stearic acid (18:0) to oleic (18:1-9) is catalyzed by steroyl-ACP Δ9 desaturase that introduces a double bond at the 9^{th} carbon of the fatty acyl chain. Membrane bound desaturases introduce double bonds into fatty acyl chain, which are esterfied to the glycerol moiety, such as phosphatidylcholine and phosphatidylethanolamine, two major phospholipids of the membrane (Shanklin *et al*., 1998). Whereas, in animals all membrane desaturases are known only to use CoA linked substrates, rather than ACP (Pereira *et al*., 2003).

Soluble desaturases are known to have two conserved histidine motifs, while membrane desaturases have three conserved histidine motifs and four transmembrane domains. As previously mentioned, soluble desaturases are only found in choloroplasts of higher plants and have shown little or no evolutionary relationship with the more widely distributed membrane desaturases (Shanklin *et al.*, 1998)

Membrane bound desaturase enzymes can be classified into three major classes based on the regioselectivity.

Delta (Δ) desaturase or "**front end desaturases**": They introduce a double bond between the existing double bond and the carboxyl group of a fatty acyl chain. Examples include Δ4 desaturase, Δ5 desaturase and Δ6 desaturase (Napier *et al*., 1999).

Omega (ω) desaturases: They introduce a double bond between the existing double bond and the methyl group of a fatty acid chain. Examples of these include the ω3 desaturases, Δ15 desaturase and Δ17 desaturase.

v+3 Desaturases: This group of desaturases primarily introduces a double bond at three carbons away from a reference double bond. Examples include the so-called "Δ12 desaturase" which introduces a double bond at the 12th position in reference to the existing Δ9 double bonds (Meesapyodsuk *et al*., 2007).

All these membrane desaturases contain the characteristic three histidine-rich motifs, which aid in the binding of the catalytic iron (Fe) that serves as an electron donor. Mutagenesis analysis of these conserved motifs indicated that the histidine amino acids within the motifs are essential for

the desaturase activity (Shanklin *et al*., 1994). Besides the three histidine motifs, front-end desaturases contain an additional cytochrome b5 domain, which is fused to the N-terminal end of the desaturase protein (Napier *et al*., 2003).

Since desaturation steps are considered the rate-limiting step for the VLCPUFA biosynthesis, many previous studies have focused on the isolation and characterization of genes encoding these desaturases. Genes encoding different types of desaturases have been isolated from many living organisms including Δ4 desaturase from *Thraustochytrium sp.* (Qiu *et al*., 2001); Δ5 desaturase from *Mortierella alpina* (Knutzon *et al*., 1998; Michaelson *et al*., 1998a), *Caenorhabditis elegans* (Michaelson *et al*., 1998b) and human (Cho *et al*., 1999a); Δ6 desaturase from *M. alpina* (Huang *et al*., 1999; Sakuradani *et al*., 1999; Sakuradani and Shimizu, 2003), *Cyanobacteria* (Reddy *et al*., 1993), *Borage officinals* (Sayanova *et al*., 1997), *C. elegans* (Napier *et al*., 1998), *Physcomitrella patens* (Girke *et al.*, 1998); Δ6/Δ5 desaturase fusion protein from *Danio rerio* (Hastings *et al*., 2001); Δ8 desaturase from *Euglena gracilis* (Wallis and Browse, 1999); ω3 fatty acid desaturase from *Claviceps purpurea* (Meesapyodsuk *et al*., 2008), cyanobacteria (Sakamoto *et al*., 1994), *C. elgans* (Spychalla *et al*., 1997) and *Phytophthora infestans* (Wu *et al*., 2005).

2.5.2 Δ6 Desaturases

The Δ6 desaturase is a key enzyme in the biosynthesis of highly unsaturated fatty acids such as ARA, EPA and DHA. ARA, the major metabolite of the ω6 pathway, and DHA the major product of the ω3 pathway, are dependent on the Δ6 desaturation of LA and ALA. In a sense one could say Δ6 desaturase acts as a gateway for fatty acids to enter into the latter desaturation and elongation steps.

The Δ6 desaturase is a "front end desaturase" as it is responsible for the insertion of a carbon double bond at the 6^{th} position from the carboxylic end of a fatty acid with an existing double bond at the 9^{th} carbon, such as LA or ALA. The isolation of the PUFA-specific Δ6 desaturase was first reported from the cyanobacterium *Synechocystis sp.* strain PCC-6803 through gain of functional cloning by expression of *Synechocystis sp.* genomic library in *Anabaena sp.* strain PCC-7120, a cyanobacterium lacking a Δ6 desaturase (Reddy *et al*., 1993). Subsequently, the

first plant Δ6 desaturase was identified from *Borage officinalis* by degenerate RT-PCR. When it was expressed in tobacco, the transgenic plants accumulated the Δ6 desaturated products, γ-linolenic acid (GLA) and stearidonic acid (SDA), accounting for 20% of the total fatty acids. Besides this astonishing result, authors noted that this desaturase contained the N-terminal extension of the normal membrane desaturases that showed homology to cytochrome b5. This small microsomal protein acts as an electron donor in a number of biochemical reactions, including fatty acid desaturation and hydroxylation. The unique structure of the electron donor being physically linked to the enzyme distinguishes it from other desaturases like ω desaturases. Interestingly, this N-terminal cytochrome b5 fusion had been previously identified in desaturase – like sequences from sunflower and *Brassica*, but no function was assigned to these sequences at that time. Due to the fact that both these species do not produce Δ6 desaturated fatty acids, it was unlikely that these homologous sequences have similar functions as the *Borage* Δ6 desaturase. Mutagenesis analysis was performed with the systematic deletion of residues 121 - 140 at the N-terminal region of the Δ6-desaturase enzymes and subsequent assaying of the desaturase activity in transgenic Arabidopsis plants. In addition, a more precise approach was taken to examine the histidine residues in that region, by substituting them with alanine residues. The result showed that truncated and the mutated enzymes exhibited no activity indicating that this region was essential for the activity of the enzyme (Sayanova *et al.*, 1997). Since the initial identification of the Δ6 desaturase gene, orthlogs have now been identified from many species including *Caenorhabditis elegans* (Napier *et al*., 1998), *Physcomitrella patens* (Girke *et al*., 1998), *Mus musculus* (Cho *et al*., 1999b), *Homo sapiens* (Marquardt *et al*., 2000), *Pythium irregulare* (Hong *et al*., 2002), *Echium plantagineum* (Zhou *et al*., 2006), *Mortierella alpina* (Huang *et al*., 1999). All these Δ6 desaturases contain cytochrome b5 fusion at the N-terminus.

In addition to the cytochrome b5 region, front-end desaturases also have another unique feature found at the 3rd histidine box where the 1st histidine is replaced with a glutamine amino acid. Experiments were done to artificially re-create the histidine residue, but this resulted in the generation of a non-functional enzyme, implying that the glutamine residue is essential for the proper function of front-end desaturases, which separates them from the consensual methyl-directed desaturases.

It has been known from previous studies that many plant and microbial species produce Δ6 desaturated fatty acids. For instance, *Primula* plants accumulate substantial amounts of stearidonic acid (SDA) and GLA. A Δ6 desaturase was cloned from this specie (Sayanova *et al*., 2006) and functional characterization of this enzyme showed that it had strong substrate specificity for ALA rather than LA, while the Δ6 desaturases in other species like *Spirulina* and *Mucor rouxii* prefer LA as substrates (Laoteng *et al*., 2000). It has been implied that the desaturation occurs in the fatty acid (LA or ALA) acylated to sn-2 position of phospholipids (Domergue *et al*., 2003). This idea was later confirmed by (Kajikawa *et al.,* 2004) when they assayed Δ5 desaturase and Δ6 desaturase from liverwort and revealed a large percentage of the desaturated and elongated fatty acids were found at the sn-2 position of phosphatidylcholine.

2.5.3 Elongases

Elongases are the other major component in the fatty acid modification pathway. These enzymes are responsible for the systematic addition of a two-carbon unit to the carboxyl end of a long chain fatty acid. They are found in many different organisms where different elongation systems exist with various substrate specificities with regards to the fatty acid chain length and degree of unsaturation.

In both plants and animals the elongase system is composed of four enzymes: a condensing enzyme beta-ketoacyl CoA synthase (KCS), a beta-ketoacyl CoA reductase (KCR), a beta-hydroxyl CoA dehydratase, and a trans-2-enoyl CoA reductase (Leonard *et al*., 2004). Fatty acid elongation systems in plants and animals are fundamentally different from their fatty acid synthesis systems. In plants a fatty acid synthase (FAS) comprises four separate enzymes that act coordinately with one another during the fatty acid synthesis starting from acetyl-ACP, which occurs within the plastid of plants. In animals this FAS complex is a dimer of two large molecules with multifunctional domains. The fatty acid synthesis from acetyl-ACP to long chain acyl-ACP occurs in the cytosol of animals. On the other hand, the fatty acid elongation of long chain fatty acids (16 and 18C) in plants and animals occurs in the ER using a discrete four-enzyme complex. It is also noteworthy that the initial fatty acid synthesis in the cytosol of animals and in the plastid of plants uses acyl chain linked to ACP moiety as substrates, while fatty acid elongation uses acyl-CoA as the substrate.

Figure 2.6 illustrates the four-step process of the fatty acid elongation that occurs in plants, microbes and animals, described below:

a. Fatty acid elongation is initiated by the condensation of malonyl-CoA with a long chain acyl-CoA, yielding the products beta-ketoacyl-CoA and carbon dioxide, catalyzed by the enzyme keto-acyl-CoA synthase (KCS).
b. Beta-ketoacyl-CoA is an acyl moiety lengthened by a two-carbon unit. This product is then reduced to beta-hydroxylacyl-CoA by the enzyme ketoacyl reductase (KCR).
c. Next beta-hydroxylacyl-CoA is dehydrated by the 3-hydroxylacyl-CoA dehydratase enzyme, yielding enoyl-CoA and water.
d. A second reduction catalyzed by enoyl-CoA reductase then occurs to produce the final elongated fatty acyl-CoA product (Leonard *et al*., 2004).

As shown in Figure 2.6, the process of fatty acid elongation requires four different enzymes to work coordinately. One would question which one of these four enzymes is responsible for the substrate specificity and rate limiting. Many scientists have asked this same question, but unfortunately the purification of these membrane bound enzymes was a very daunting task, which makes the biochemical study of these enzymes very difficult. Nevertheless, the recent genetic studies indicate that the condensing enzyme, keto-acyl synthase (KCS), of the elongation system determines the substrate specificity

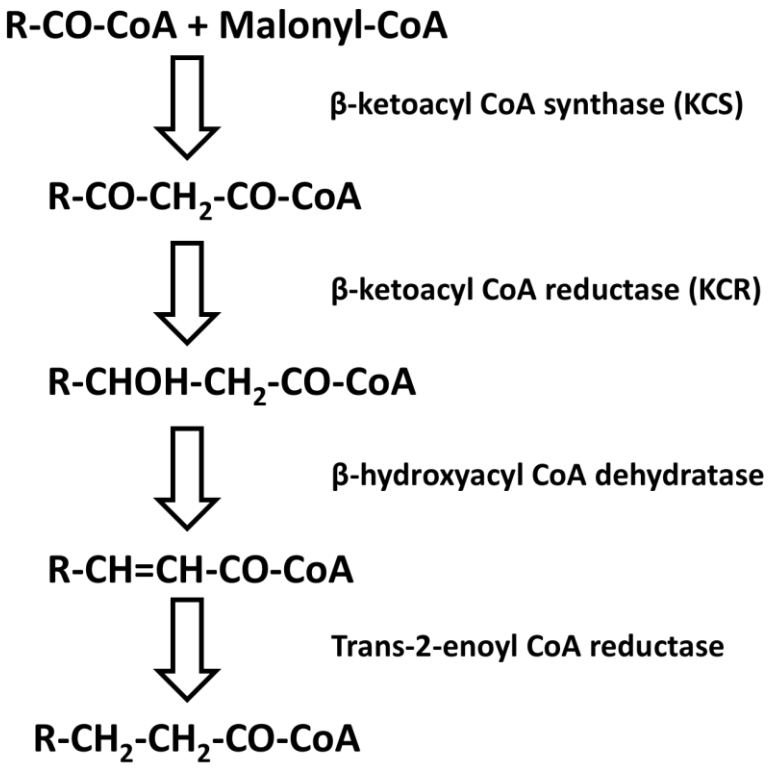


Figure 2.6 Fatty acid chain elongation reactions.

with regards to fatty acid chain length and degree of unsaturation, and the condensation step is the rate-limiting process and different condensing enzymes apparently share the same set of reductases and dehydratases (Leonard *et al*., 2002). Thus, in the last decade main focus has been on the condensing enzyme, also known as elongase. Many different genes coding for KCS or fatty acid elongase (FAE) have been cloned and characterized from various organisms including *Saccharomyces cerevisiae* ELO1 (Toke and Martin, 1996), ELO2, ELO3 (Oh *et al*., 1997); *Homo sapiens* (Leonard *et al*., 2002), *Rattus norvegicus* (Inagaki *et al*., 2002), *Mus musculus* (Tvrdik *et al*., 2000), *P. patens* (Zank *et al.*, 2002), alga *Isochrysis galbana* (Wallis and Browse, 1999), fungus *M. alpina* (Parker-Barnes *et al*., 2000). Based on their primary protein sequences and substrate specificity, elongase enzymes are classified into two different groups, saturated/mono-unsaturated fatty acid (MUFA) elongase and poly-unsaturated fatty acid (PUFA) elongase (Hashimoto *et al*., 2008).

2.5.4 Δ6 Elongases

Δ6 elongase is an enzyme catalyzing the elongation of both ω6 and ω3 Δ6 desaturated fatty acids by a two-carbon unit. In the ω6 pathway, the Δ6 elongase enzyme is responsible for the elongation of γ-linolenic acid to dihomo-γ-linolenic acid, which can be further desaturated to ARA. In the ω3 fatty acid biosynthesis pathway, Δ6 elongase enzyme catalyzes the elongation of SDA into ETA that also can be further desaturated to EPA.

One of the very first Δ6 elongase was cloned from the fungus *M. alpina* by Parker-Barnes *et al*. in 2000 and in the same year another Δ6 elongase was identified from *C. elegans* (Beaudoin *et al*., 2000). The two enzymes showed high sequence similarity to the yeast ELO family where ELO1 was shown to elongate long chain saturated and monounsaturated fatty acids (18C – 20C), and ELO 2 and ELO3 were shown to elongate very long chain saturated and monounsaturated fatty acids (20C – 26C). Since then, many Δ6 elongase have been identified from various organisms including fungi (Meyer *et al*., 2004; Parker-Barnes *et al*., 2000), algae (Domergue *et al*., 2005; Iskandarov *et al*., 2009; Meyer *et al*., 2004) and animals (Leonard *et al*., 2002; Meyer *et al*., 2004).

2.6 Reconstitution of the VLCPUFAs biosynthetic pathway in yeast and plant

Heterologous expression of genes in yeast *S. cerevisiae* has proven to be a valuable tool for the functional characterization of genes involved in the production of VLCPUFAs. *Saccharomyces cerevisiae* serves as the model organism for the development of metabolic engineering strategies to produce VLCPUFAs as well, as it is relative easy to co-express multiple genes in the yeast for the reconstitution of the partial or entire metabolic pathways. In addition, the result of reconstituting the pathways in yeast is effectively indicative of that in other heterologous expression system such as oilseed plants, thereby allowing for early assessment of metabolic problems that may occur later in transgenic plants.

The first reconstitution of γ-linolenic acid in yeast was achieved with the co-expression of Δ12 desaturase and Δ6 desaturase from *M. alpina* (Huang *et al*., 1999). Since this initial achievement, many others were followed with reconstitution of other parts of the pathway. The production of ARA and EPA in yeast was achieved by co-expression of Δ5 desaturase and Δ6 elongase from *M. alpina* with exogenous fatty acid supplementation (Huang *et al*., 1999; Parker-Barnes *et al*., 2000).

Later, Beaudoin *et al*. (2000) and Domergue *et al*. (2002) independently reported the synthesis of small amounts of ARA and EPA in yeast through the co-expression of three genes, beginning with the one encoding Δ6 desaturase in presence of precursor fatty acids. Through a series of experiments, four major conclusions were drawn: 1) the feasibility of the reconstruction of the VLCPUFAs pathway in yeast, 2) Heterologous condensing enzyme or elongase is compatible with other components of the endogenous elongase complex in the host, 3) The VLCPUFA enzymes were not specific for one pathway, but rather operated in both the ω3 and ω6 pathways, and 4) Unexpected products were formed from the side reactions of the desaturases and elongases acting non-specifically.

Domergue *et al*. (2003) revisited the reconstitution of the ARA biosynthetic pathway. The detailed analysis of desaturation and elongation reactions in yeast transformants confirmed that the elongation occurs in acyl-CoA pool, while the phospholipid-linked fatty acyl chains were the substrates for the desaturases. The poor yield of ARA was attributed to limited availability of the

elongase substrate. The authors reasoned that newly synthesized GLA could be effectively transferred from the phospholipids to the neutral lipids by phospholipids diacylglycerol acyltranferase activities converting PC to DAGs, thus by-passing the acyl-CoA pool. The low amount of GLA/SDA in the acyl-CoA pool was attributed to the inability of the yeast host to efficiently transfer these acyl moieties from the phospholipids (Domengue *et al*., 2003).

Meyer *et al*. (2004) reconstituted the partial DHA pathway in yeast commencing with the Δ6 elongation step. A Δ5 desaturase from the diatom *Phaeodactylum tricornutum* and a Δ4 desaturase from protist *Euglena gracilis* were co-expressed with the bifunctional Δ6/5 elongase from the fish *Oncorhynchus mykiss* or with Δ6 elongase from the diaotom *T. pseduomona* and a Δ5 elongase from *Osteococcus tauri*. Reconstitution in both gene sets resulted in production of a small amount of DHA in presence of exogenous Δ6 desaturated precursors (Meyer *et al*., 2004).

Many reconstituting experiments have produced VLCPUFAs in *S. cerevisiae,* but all require exogenous supplementation of precursor fatty acids to the yeast. Yawaza *et al*. (2007) was the first to reconstruct the entire DGLA pathway without exogenous fatty acid supplementation by taking advantage of a Δ12 desaturase from *Kluyveromyces lactis* and a Δ6 desaturase and a Δ6 elongase from *Rattus norvegicus* (Yawaza *et al*., 2007).

After the successful reconstitution of the VLCPUFA pathway in yeast, researchers naturally moved their attention to the reconstruction of these pathways in plants. Abbadi *et al*. (2004) transformed tobacco and flax with binary vectors carrying different combinations of Δ6 desaturase from *P. patens, B. officinalis,* and *P. tricornutum*, Δ6 elongases from *P. patens* and *C. elegans* and Δ5 desaturase from *M. alpina* and *P. tricornutum*, all under the control of seed specific promoters. However, only very small amounts of VLCPUFAs were produced in the seeds of transgenic plants. The low level production was hypothesized to be due to the inefficient elongation of 18 carbon precursors, as analysis of the acyl-CoA pool revealed limited amounts of these precursors present in the acyl-CoA pool. When acyl-CoA and malonyl-CoA were supplemented to the microsomes isolated from the transgenic developing seeds, the substrate was efficiently elongated. This data excluded the possibility that low level of elongation efficiency is due to the low activity of the transgenic elongase gene.

Reconstitution of DHA biosynthetic pathway in oil seed crop Indian mustard (*Brassica juncea*) was achieved in our lab with a series of binary vectors carrying three to nine genes, with each gene under the independent control of a seed-specific promoter. The stepwise engineering process offered the opportunity to observe the individual effects of each gene in the VLCPUFA biosynthetic pathway and provided very invaluable information pertaining to the intermediate steps of the complex pathway (Wu *et al*., 2005). *Brassica juncea* plants, naturally rich in linolenate acid (18:3ω3), were initially transformed with three genes constituting the minimal set of genes necessary for the biosynthesis of 20 carbon VLCPUFAs. This construct consists of a Δ6 desaturase from *P. irregularae*, Δ6 elongase from *P. patens* and a Δ5 desaturase from *Thraustochytium sp*. The transgenic seeds were able to accumulate an average 7% ARA and smaller amounts of EPA. Addition of a heterologous plant Δ 12 desaturase from *Calendula officinalis* resulted in enhanced conversion of 18:1ω9 to 18:2ω6, which consequently increased the product of ARA in the seeds (12%). Based on the observed results that elongation was the rate-limiting step, an additional Δ6 desaturase was added. To increase the yield of ω3 fatty acids a ω3 desaturase from *Phytophthora infestans* was introduced, which resulted in effective conversion of the ARA to EPA. Upon the adequate accumulation of EPA, a Δ4 desaturase, putative lysophosphatidylcholines acyltransferase (LPCAT) from *Thraustochytrium sp.* and Δ5 elongase from *O. mykiss* was introduced to completely reconstitute the entire DHA pathway. This gene set represents one of the largest numbers of genes expressed from a single construct in plants. The average yield of DHA was recorded to be ~0.2% (Wu *et al*., 2005).

Another experiment worth mentioning is the reconstitution of the alternative pathway in plant. Qi *et al*. (2004) sequentially transformed the model plant *Arabidopsis thaliana* with genes encoding an algal Δ9 elongase from *I. galbana*, Δ8 desaturase from the protist *E. gracilis* and a fungal Δ5 desaturase from *M. alpina*. This alternative pathway was believed to be able to alleviate the problem with inefficient Δ6 elongation in the common pathway. However, production of VLCPUFAs in seeds was still very low, although transgenic leaves accumulated 7% ARA and 3% EPA (Qi *et al.*, 2004), suggesting that additional factors are required for a high level production of VLCPUFAs in heterologous systems.

3.0 Study 1: VLCPUFA analysis of the Entomopathogenic Fungi

3.1 Abstract

With the aid of gas chromatography and GC/MS, several species of entomopathogenic fungi previously reported to produce very long chain polyunsaturated fatty acids (VLCPUFAs) were analyzed to confirm the presence of Δ6 desaturated products, γ- linoleic acid (GLA, 18:3-6,9,12) and stearidonic acid (SDA, 18:4–6,9,12,15), and Δ6 elongated products dihomo-γ-linolenic acid (DGLA, 20:3-8,11,14) and eicosatetraenoic acid (ETA, 20:4-8,11,14,17) as well as their downstream products such as arachidonic acid (ARA, 20:4-5,8,11,14) and eicosapentaenoic acid (EPA, 20:5-5,8,11,14,17) which are Δ5 desaturated products of DGLA and ETA, respectively. Results showed *Conidiobolus obscurus* and *Conidiobolus thromboides* produced the greater amount of Δ6 desaturated and elongated products which were mainly located in phospholipids. These two fungal species were thus selected for further cloning studies.

3.2 Hypothesis

Fatty acid structure and compositions of living organisms are indicative of metabolic pathways and corresponding enzymes operating within the organism. We anticipate these entomopathogenic fungi could produce large amounts of VLCPUFAs as previously shown (Tyrrell, 1967). As analytical methods have evolved tremendously since 1960s, it is therefore necessary to reconfirm the results by current gas chromatography. If these entomopathogenic fungi indeed produce large amounts of VLCPUFAs as previously reported, they could then be used as potential gene sources for desaturase and elongase involved in the biosynthesis of these fatty acids.

3.3 Experimental approach

3.3.1 Organisms and growth

Five entomopathogenic fungi were kindly provided by Dr. Richard Humber of Robert W. Holley Center for Agriculture and Health, Ithaca, NY 13853-2901 (USA) including *Conidiobolus thromboides* (ARSEF120), *Conidiobolus obscurus* (ARSEF74), *Entomophora conglomenta* (ARSEF2273), *Batkoa spp.* (ARSEF3131), and *Batkoa gigantea* (ARSEF215). To analyze their

fatty acid composition, five fungi were grown as previously described with slight modification (Tyrell, 1967). The mycelium was inoculated in a quarter strength of Sabourad Dextrose Media (SAB) (10 g/L) containing 5 g/L dextrose, 2.5 g/L bactopeptone and 2.5 g/L yeast extract, and grown at 22°C for 10 days, with shaking at 180 rpm. The cells were subsequently harvested by vacuum filtration and washed twice with 20 mL of sterile water. After freeze-drying, the cells were stored at -80°C until use for fatty acid analysis and gene cloning.

3.3.2 Fatty Acids Analysis

The fatty acids were directly transmethylated with 2 mL of 3N methanolic HCl at 80°C for 2 hours. After transmethylation process, the sample was cooled down at room temperature before adding 1 mL of 0.9% NaCl and 2 mL of hexane. The sample was then mixed and centrifuged at 2,400 rpm for 5 minutes for phase separation. Hexane phase containing fatty acid methyl esters (FAMEs) were removed and dried under N_2. After drying, the sample was resuspended in 400 µl of hexane and placed in a GC auto-sampler vial for GC analysis. Two µl of the sample total FAMEs was analyzed on an Agilent 6890N gas chromatograph equipped with a DB-23 column (30m) with 0.25-µm-film thickness (J&W Scientific, Mississauga, Ontario). The column temperature was maintained at 160 °C for 1 min, then raised to 240 °C at a rate of 4 °C/min (Reed *et al*., 2000). The areas of chromatographic peaks were calculated for relative amounts of FAMEs.

For lipid class analysis, *C. obscurus* and *C. thromboides* were grown in 40 mL of the quarter strength SAB media at 22°C for 10 days as described by Dr. Humber of ARSEF. The biomass was collected through vacuum filtration and washed twice with 20 mL of distilled water and air-dried appropriately. The sample was then added with 7 mL of 2:1 chloroform: methanol (v/v) mixture and homogenized for two minutes. The homogenized solution was centrifuged at 2400 rpm for 5 minutes. Ideally, the sample should separate into three different layers: (a) top water layer, (b) middle cell debris layer, and a (c) bottom solvent layer containing the lipids (Aitzetmuller *et al*., 1992).

The bottom layer was carefully removed into a new tube and dried under N_2 to prevent any

oxidation of the lipid product. The dried lipid was resuspended in an appropriate volume of chloroform to achieve a final concentration of 20 μg/μL.

To fractionate the different lipid classes, the total lipid extract was resolved on silica G-25 thin layer chromatography and developed with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) for neutral lipids and with chloroform/methanol/ acetic acid/water (100:40:12:2, vol/vol/vol/vol) for phospholipids, respectively. Once the solvent front reached within two centimeters of the top of the plate, the developed plate was air dried and sprayed with the lipid staining solution (5mg primuline in 80:20 acetone:water, v/v). Lipid staining was observed under an UV transluminator (Aitzetmuller *et al*., 1992).

The spots corresponding to each lipid classes in reference to lipid standards were then scratched off the silica plate and directly transmethylated as described above.

3.4 Results

Fatty acid profiles of five entomopathogenic fungi determined by GC are shown in Figure 3.1. Identities of each FAMEs in the chromatogram were determined based on their retention times and mass spectra in comparison with those of authentic standards (Nu-Chek, Elysian, Minnosota). The relative amount of each fatty acid was calculated and used for the comparison between fungal species (Figure 3.2).

As shown in Figure 3.1, each fungus produced its own unique the fatty acid profile, which could be used to differentiate from each other. *Batkoa spp.* was able to accumulate very large amounts of saturated C16:0 fatty acids (58%) and low levels of long chain and VLCPUFAs including DGLA, ETA, ARA and EPA (<10%).

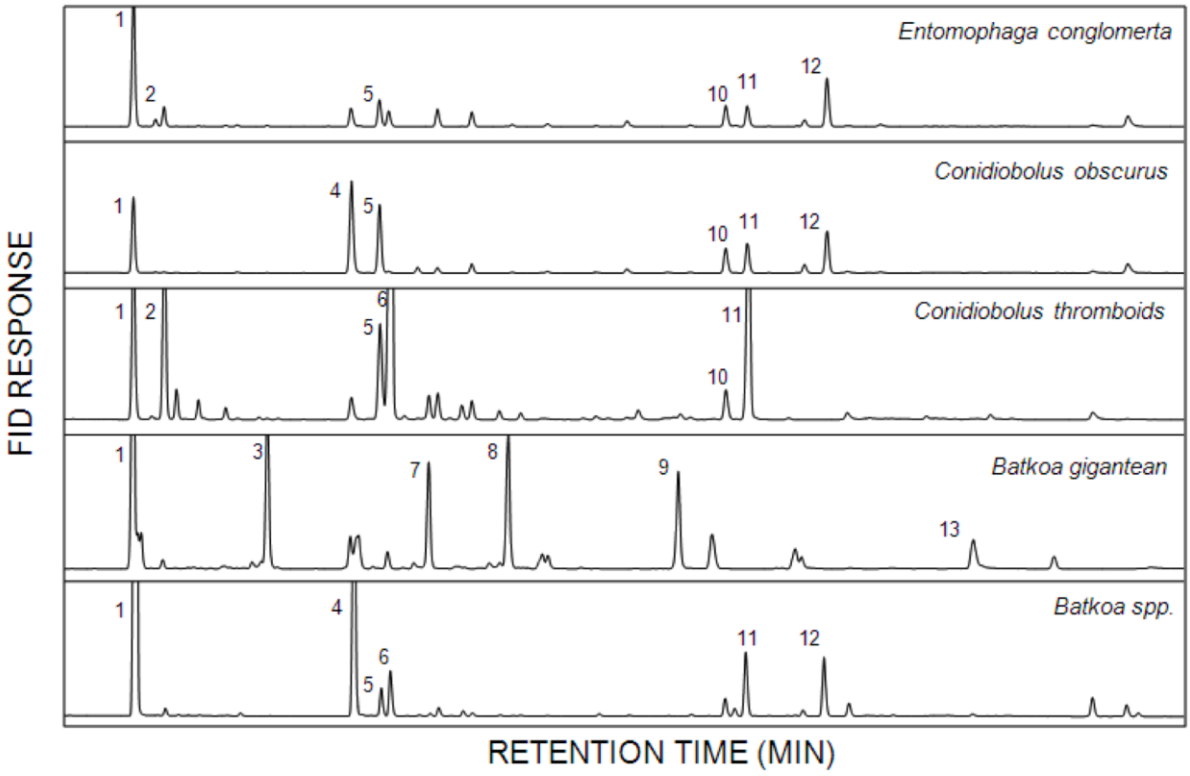


Figure 3.1 GC analysis of total FAMEs prepared from cultures of entomopathogenic fungi. The fatty acids are denoted as: **(1)** 16:0; **(2)** 16:1-9; **(3)** 17:0; **(4)** 18:0; **(5)** 18:1-9; **(6)** 18:1-11; **(7)** 18:2-9,12; **(8)** 18:3-9,12,15; **(9)** 20:2-11,14; **(10)** 20:3-8,11,14; **(11)** 20:4-5,8,11,14; **(12)** 20:5-5,8,11,14,17; **(13)** 24:0.

Table 3.1 The fatty acid compositions of selected entomopathogenic fungi.

	Saturated/ monounsaturated fatty Acids				Long chain polyunsaturated fatty acids (PUFAs)					Very long chain PUFAs (VLCPUFAs)			
	16:0	**18:0**	**18:1-9**	**18:1-11**	**18:2**	**GLA**	**ALA**	**SDA**	**20:2**	**DGLA**	**ETA**	**ARA**	**EPA**
A	29.2	4.5	6.5	3.9	4.2	3.5	0.0	0.0	0.0	5.2	1.7	5.2	12.2
B	17.7	24.9	17.1	0.0	1.4	2.4	0.0	0.0	0.0	7.0	2.4	8.2	11.7
C	11.1	1.6	6.9	39.0	1.7	1.1	0.0	0.0	0.0	2.1	0.0	17.0	0.0
D	36.7	3.4	6.0	1.8	11.1	0.0	14.9	2.1	11.4	5.2	1.3	0.0	0.0
E	58.3	16.8	1.5	2.5	0.5	0.0	0.0	0.0	0.0	1.1	0.4	4.0	3.7

A) *Entomophaga conglomerta*, B) *Conidiobolus obscurus*, C) *Conidiobolus thromboides*, D) *Batkoa gigantean*, E) *Batkoa spp*. Values are represented as TFA% (weight percentage of total fatty acids).

Batkoa gigantean, a relative species to *Batkoa spp.* could accumulate substantial amounts of α-linolenic acid (18:3-9,12,15) (~15%), but very low levels of VLCPUFAs. It did not produce any ARA and EPA, implying this fungal species might lack any Δ5 desaturases for synthesis of the two Δ5 polyunsaturated fatty acids.

Conidiobolus thromboides, C. obscurus and *E. conglomerta* are three entomopathogenic fungi that could produce substantial amounts of VLCPUFAs (>20%). *Conidiobolus thromboides* was able to produce a large amount of ω6 fatty acids such as DGLA and ARA; whereas, *C. obscurus* and *E. conglomerta* were capable of synthesizing both ω3 and ω6 VLCPUFAs such as EPA and ARA. Therefore, it could be expected that all three fungal species might contain at least an active Δ6 elongase and Δ6 desaturase as well as an active Δ5 desaturase, which are responsible for synthesis of the final products, ARA and EPA.

The fungal species capable of producing the largest amount of Δ6 desaturated products (D6DP) and Δ6 elongated products (D6EP) were selected as potential gene sources for the identification of Δ6 desaturase and Δ6 elongase. The D6DP was calculated by adding all the products downstream Δ6 desaturation, which include GLA, DGLA, ARA, SDA, ETA, EPA, while the D6EP value was calculated by adding all the downstream products of Δ6 elongations, which include DGLA, ETA, ARA and EPA. Based on the calculation, *C. obscurus* produced the greatest amount of D6DP (31.7%) and D6EP (29%) and was thus selected as our first choice for the gene source for Δ6 desaturase and Δ6 elongase. In addition, *C. thromboides* was also selected as another potential candidate, as it produced the highest amount of arachidonic acid among the five fungal species, suggesting there might be a very effective ω6 pathway operating in this fungus (Table 3.1).

Fatty acid analysis of fungal species by means of direct transmethylation of the biomass only provides a simple overview of the sample's total fatty acid composition. To obtain better understanding of the fatty acid distribution among different lipid classes, fatty acid composition of glycerolipids, such as neutral lipid triacylglycerols (TAGs) and polar lipid phospholipids was analyzed by gas chromatography in conjunction with thin layer chromatography (TLC). Figure

3.2 shows the fatty acid composition of the neutral lipid TAGs and polar phospholipids separated by TLC plate in *C. obscurus* and *C. thromboides*, respectively. Because a large fraction of the VLCPUFAs were found located in the phospholipids, making up ~25% in *C. thromboides* and ~50% in *C. obscurus*, the fatty acid compositions of two major phospholipid classes PC (phosphatidylcholine) and PE (phosphatidylethanolamine) were also analyzed (Figure 3.3).

As shown in Figure 3.2, the predominate fatty acid in the TAG fraction of *C. obscurus* was stearic acid (18:0) which accounts for more than 50% of the total fatty acids, while ARA and EPA each accounts for less than 10% in the neutral lipid fraction. On the other hand, ARA and EPA were two major fatty acids in the phospholipid fraction; together they made up approximately 40% of the total fatty acids. In contrast, stearic acid was less than 5% of the total fatty acids in the phospholipid fraction. *Conidiobolus thromboides* did not produce any EPA in either TAG or phospholipid fractions, which is consistent with the result from the fatty acid analysis of the total lipids. Similar to *C. obscurus*, C. *thromboides* contained a high level of ARA in the phospholipid fraction (more than 25%). However, in the TAG fraction, vaccenic acid (18:1-11) other than stearic acid was the predominate fatty acid accounting for approximately 25% of the total TAG fatty acids.

As VLCPUFAs were mostly located in the phospholipid fraction, the fatty acid composition of two major phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were further determined in the two fungal species. As shown in Figure 3.3, only four major fatty acids (16:0, 16:1-9, 18:1-11 and ARA) were detected in PC and PE fractions of *C. thromboides* and ARA was the predominate VLCPUFA accounting for approximately 33% of total fatty acids in PC and 30% in PE. The PC fraction contained a much lower level of vaccenic acid, but a higher level of palmitic acid (16:0) compared to the PE fraction. In comparison with *C. thromboides*, C. *obscurus* comprises a wider range of fatty acids in both PC and PE fractions where major fatty acids were 16:0, 18:0, ARA and EPA. PE contains higher levels of ARA and EPA, albeit little GLA and DGLA, two Δ6 polyunsaturated fatty acids. While PC contains less ARA and EPA, it comprised fair amounts of GLA and DGLA, together they made up approximately 10 % of the total fatty acids in the fraction.

3.5 Discussion

Previous reports have indicated entomopathogenic fungi such as *Conidiobolus spp, Entomophora spp.* and *Batkoa spp.* could produce large amounts of VLCPUFAs (Tyrrell, 1967). With the aid of current gas chromatography and GC/MS technologies, five species of these entomopathogenic fungi have been selected for the analysis of their VLCPUFAs. The result showed that all these fungi, particularly *C. obscurus* and *C.thromboides* produced large amounts of VLCPUFAs such as arachdonic acid and eicosapentaenoic acid which are downstream products of Δ6 desaturated and Δ6 elongated products. *Conidiobolus obscurus* produced a wide range of both ω3 and ω6 VLCPUFAs such as DGLA, ETA, ARA and EPA, while *C. thromboides* produced only ω6 VLCPUFAs such as DGLA and ARA. Presence of high amounts of VLCPUFAs is indicative of active metabolic pathways and corresponding enzymes operating in these fungi. Therefore, they can be good gene sources for desaturase and elongase involved in the biosynthesis of VLCPUFAs.

Interestingly, VLCPUFAs produced in these two fungal species were mainly found in phospholipids particularly, phosphatidylethanolamine; whereas the storage neutral lipid triacylglycerol contained lower amounts of VLCPUFAs. The biosynthesis of VLCPUFAs involves alternating desaturation and elongation. ARA and EPA are two Δ5 desaturated fatty acids in the ω6 and ω3 pathways and their biosynthesis are generally believed to occur in phospholipids. Therefore, high level VLCPUFAs in phospholipids rather than in triacylglycerols implies that *Conidiobolus spp.* have developed an active system to synthesize these VLCPUFAs, but lack an efficient mechanism to channel these fatty acids to the storage lipids. Diacylglycerol acyltransferase (DGAT) catalyzes the committed step of the triacylglycerol biosynthesis using diacylglycerol and fatty acyl-CoA as substrates. DGAT is the only enzyme in the triacylglycerol-biosynthetic pathway, which is not shared with the biosynthesis of phospholipids. Based on the primary protein sequence, DGATs identified from eukaryotes can be classified into two types. In yeast and fungi, biosynthesis of storage triacylglycerols is mainly catalyzed by type II DGAT (DGAT2). The low level of VLCPUFAs in triacylglycerols of the two fungal species thus might be due to the low activity of their DGAT2 enzymes.

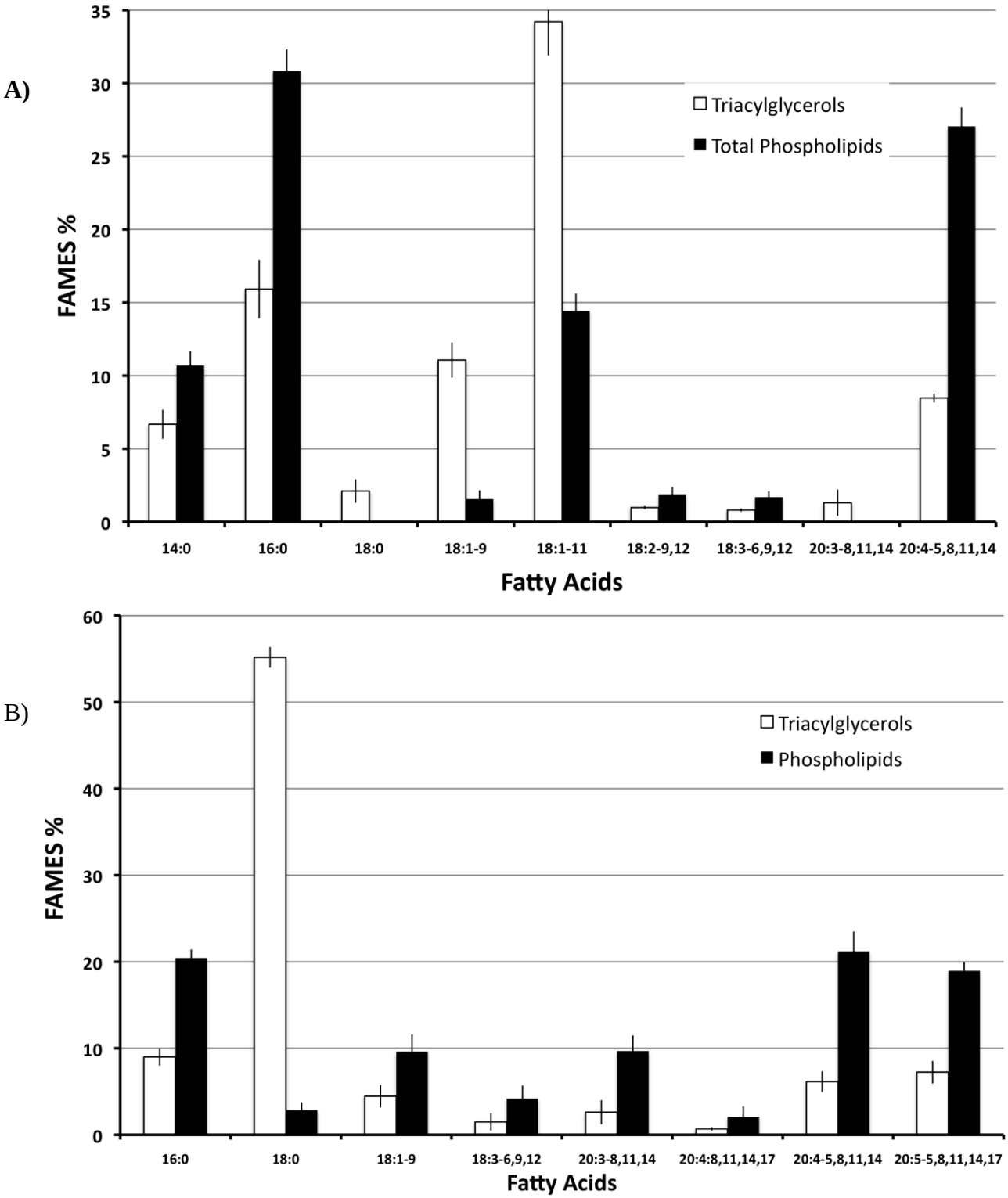


Figure 3.2 Fatty acid profile of TAGs and total phospholipids of *C. thromboides* (A) and *C. obscurus* (B).

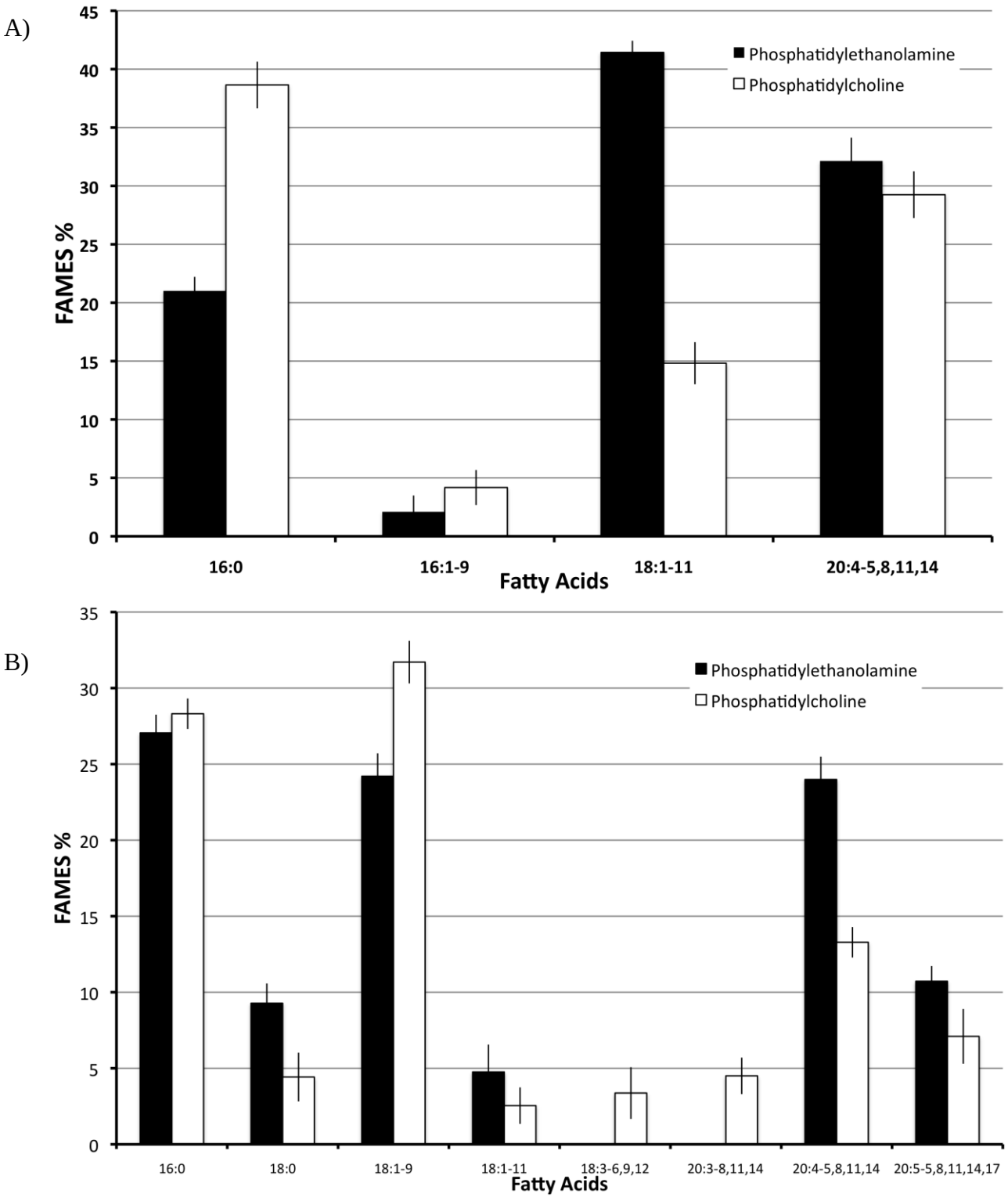


Figure 3.3 Fatty acid profiles of PC and PE of *C. thromboides* (A) and *C. obscurus* (B)

Fungi in the phylum of Zygomycota, including entomopathogenic fungi, have been reported to be able to accumulate VLCPUFAs (Weber and Tribe, 2003). These fungal species have the ability to convert sugars or other carbon sources into the polar and neutral lipids. The process of the lipid accumulation in these oleaginous fungi is triggered with the exhaustion of a nutrient from the media, usually nitrogen. With the limited supply of nitrogen, the cell proliferation slows down and the triacylglycerol is actively synthesized in the existing cells rather than dividing cells (Ratledge *et al*., 2000). More recently, the malic enzyme has been shown to play an important role in the accumulation of various lipids in oleaginous fungi such as *Aspergillus nidulans* and *Mucor circinelloides*. Experiments carried out by Ratledge's group showed the treatment of *M. circinelloides* with sesamol, the non-oil component of sesame seed oil resulted in the loss of their ability to accumulate a large amount of lipids. Compared with the untreated control accumulating 25% lipids, the treated cells produced only 2% lipids. The specific inhibition of malic enzyme activity was found to be responsible for the reduced lipid accumulation (Wynn *et al*., 1997). The mutant strain of *Aspergillus nidulans* that lacks a functional malic enzyme produced the similar result (Wynn *et al*., 1997). Malic enzyme catalyzes the conversion of malate to pyruvate producing NADPH. This reducing power generated by the malic enzyme has been considered to be critical for both *de novo* fatty acid biosynthesis and fatty acid desaturation. Thus, it would be interesting to know whether a high level of VLCPUFAs in *Conidiobolus spp.* has something to do with their malic enzymes activity.

Polyunsaturated fatty acids have been shown to play an important role in sexual development and spore germination of several filamentous fungi. In *Neurospora sp*. α-linoleate (18:3ω3) stimulates formation of fruiting bodies (Nukina *et al*., 1981). In *Mucor sp*, γ-linolenic acid is steadily increased during the germination process of spores (Laoteng *et al*., 2003) and the Δ6 desaturase gene responsible for the biosynthesis of this fatty acid is highly expressed during the spore germination (Khunyoshyeng *et al*., 2002). Entomopathagenic fungi are able to infect insects. Once the fungus is in the hemocoel of the host, it produces yeast-like hyphal bodies and wall-less protoplasts. The protoplasts, unlike hyphal bodies, are not recognized by the immune system of insects because of the lack of β-1,3 glucan in the cell walls (Tanada and Kaye, 2003).

It appears that VLCPUFAs can inhibit the synthesis of β-1,3 glucan in the protoplasts, which may allow the fungus to successfully infect the host, rapidly spread and eventually kill its host (Mackichan *et al*., 1995).

4.0 Study 2: Cloning and functional characterization of genes encoding Δ6 desaturases from *C. thromboides* and *C. obscurus*

4.1 Abstract

The genes encoding putative Δ6 desaturases (*CtD6* and *CoD6*) were cloned from *C. thromboides* and *C. obscurus* by the degenerate reverse transcriptase - polymerase chain reaction (RT-PCR) approach. Degenerate primers were designed to target the conserved regions of the Δ6 desaturases previously identified from other species. Degenerate RT-PCR amplified a portion of the Δ6 desaturases from the two fungal species. The full-length genes were obtained by the 5' and 3' rapid amplification of the cDNA ends (RACE) method. To functionally characterize the putative desaturase genes, the open reading frames (ORFs) of the cDNAs, *CtD6* from *C. thromboides* and *CoD6* from *C. obscurus* were cloned into a yeast expression vector, which were transformed into the yeast *Saccharomyces cerevisiae* (INVSc1). In the presence of exogenously fed linoleic acid and α-linolenic acid, transformants expressing *CoD6* could convert these substrates into two Δ6 desaturated fatty acids, γ-linolenic acid and stearidonic acid, indicating *CoD6* codes for a functional Δ6 desaturase able to introduce a carbon-carbon double bond at the 6^{th} position of linoleic acid and α-linolenic acid. To the contrary, transformants expressing *CtD6* did not produce any new fatty acids when fed with all potential substrates, suggesting it does not code for a functional desaturase.

4.2 Hypothesis

The presence of γ-linolenic acid (GLA), stearidonic acid (STA) or their downstream fatty acids in the *Conidiobolus spp.* implies there might exist a corresponding biosynthetic pathway for synthesis of these Δ6 desaturated products. The genes encoding Δ6 desaturase in the pathway could thus be isolated by the degenerate RT-PCR approach and function of these genes could then be determined in *Saccharomyces cerevisiae*, a well-known eukaryotic model system for functional characterization of genes in the lipid biosynthesis.

4.3 Experimental Approach

4.3.1 RNA Extraction

Total RNA was isolated from the selected fungi using the TRIZOL RNA extraction method (Invitrogen Canada Inc., Burlington, Ontario). The fungal material was homogenized into a fine powder with the aid of liquid nitrogen. The powdered biomass was added to 1 ml of Trizol reagent and chloroform. The mixture was centrifuged at 12,000 g for 15 – 20 minutes at 4°C. This process separated the mixture into 3 phases, a phenol-chloroform lower phase, an interphase and a colorless upper aqueous phase. The RNA-containing aqueous phase was removed and the RNA was precipitated with isopropyl alcohol. The pellet was washed with 75% ethanol. After the multiple washings the RNA was re-suspended in 0.5% diethylpyrocarbonate (DEPC) treated water.

4.3.2 Designing of degenerate primers for isolation of fragments of Δ6 desaturase genes from *Conidiobolus spp.*

To clone a Δ6 desaturase gene, the RT-PCR method with degenerate primers was adopted. Degenerate primers have a number of options at several positions in the sequence so as to allow annealing to and amplification of a variety of related sequences. They are generally designed for the purpose of pulling out one part of a gene, using the highly conserved regions as sites for primer binding.

To design the degenerate primers for cloning the gene encoding Δ6 desaturase from *C. thromboides* and *C. obscurus* by RT-PCR, several Δ6 desaturases previously identified from other species were aligned using the DNAstar program, MegAlign. As shown in Figure 1, the shaded regions indicate amino acid sequences that were highly conserved through many species, and thus were potential target sites for degenerate primer design.

As such several sets of degenerate primers were designed in the conserved regions (shown in Figure 4.1), which were used for the amplification of a partial cDNA sequence of Δ6 desaturase from *C. thromboides* and *C. obscurus* using RT-PCR.

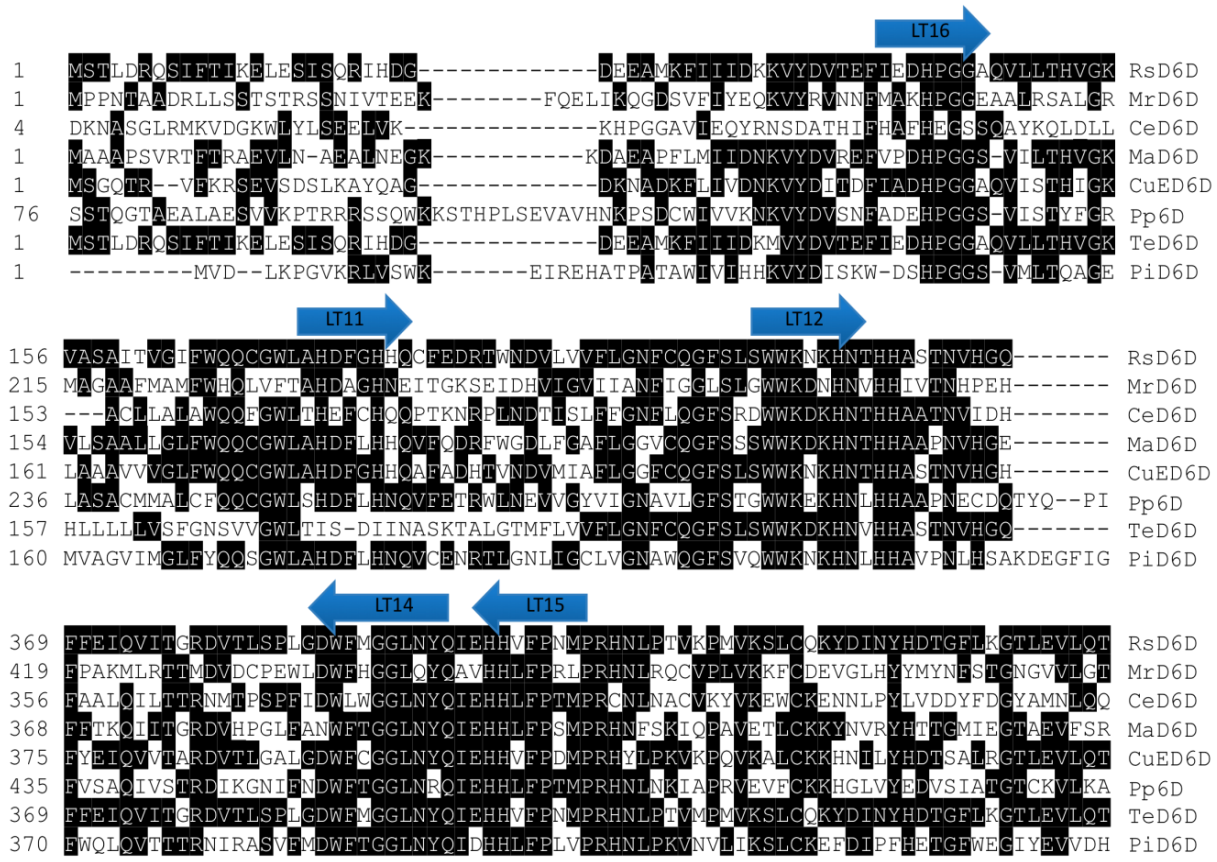


Figure 4.1 Partial amino acid alignment of Δ6 desaturases from fungi including *Mucor rouxii* (**MrD6**, AAG36959), *Mortierella alpina* (**MaD6**, AAF08685), *Rhizopus stolonifer* (**RsD6**, ABB96724), *Thamnidium elegans* (**TeD6**, AAX40418), *Cunninghamella echinulata* (**CueD6**, ABA06503), *Pythium irregulare* (**PiD6**, AAL13310), *Physcomitrella patens* (**PpD6**, CAA11032), *Caenorhabditis elegans* (**CeD6**, AAC15586). The shaded regions indicate areas of amino acid sequences that are highly conserved. Arrows indicate the regions that were selected for primer design.

The first sets of primers designed for RT-PCR were LT15 (Reverse Primer) and LT16 (Forward Primer). LT15 was designed to target the third histidine box at the conserved region (H-H-L-F-P-R-**L/M**), while LT16 was targeted to the highly conserved heme-binding domain (I-**A/E**-**D/K**-H-P-G-G). Using this set of primers an expected 1000 bp fragment should be amplified.

The second sets of primers were designed to target the other highly conserved regions, LT14 (reverse primer), LT11 and LT 12 (forward primer). LT14 is located close to the last histidine box (W-F-H-G-G-L-Q), while the primers LT11 (W-W-K-**N/D**-**N/K**-H-N) and LT12 (A-H-D-**A/F**-G-H) were designed close to the first histidine box. The primers used to isolate the partial cDNA fragments were listed in Table 4.1.

4.3.3 Degenerate RT-PCR

Degenerate RT-PCR was carried out following the protocol provided by the Superscript III kit (Invitrogen Canada Inc.).The first strand of cDNA was synthesized by the reverse transcriptase using the total RNA isolated from *C. thromboides* and *C. obscurus* as the template. Multiple PCRs were performed to obtain the fragments of Δ6 desaturase by modifying the conditions such as annealing temperature, primer concentration, enzyme concentration and salt concentrations. The PCR products were separated on a 1% agrose gel and the expected DNA fragments were cut from the gel, the products were gel purified and ligated into cloning vectors pGEM T-EASY (Promega, Nepean Ontario) or pCR4 TOPO (Invitrogen Canada Inc.). The recombinants were then transformed into *Escherichia coli (*TOP10) (Invitrogen Canada Inc., Burlington, Ontario) and sent to PBI, National Research Council of Canada, Saskatoon, Saskatchewan for sequencing. The sequencing results were searched against the NCBI non-redundant protein sequence databases using BLASTX and its defined default settings (provided at the NCBI website) to check if the cloned sequence resembles other Δ6 desaturase enzymes from other species.

4.3.4 Rapid amplification of the cDNA ends (RACE)

The RACE method was performed to obtain the missing 5’ and 3’ ends of the genes. Multiple gene-specific primers were designed from the sequence data obtained from the partial cDNA

fragment of the *Δ*6 desaturase genes. The primers used to obtain the missing 5' and 3' ends of the desaturase genes were shown in Table 4.1.

For RACE, the mRNA was extracted from the total RNA and used as a template for first and second stranded cDNA synthesis with the aid of the Marathon cDNA kit (Clontech, Mountain View, California). After the synthesis of the double stranded cDNAs, an adapter was added to the cDNA products to ensure that the amplification occurs. Using the gene-specific and adaptor primers, multiple RACE PCRs were performed to generate fragments with the expected size. These fragments were ligated into pGEM-T Easy and sequenced. The sequencing information of both cDNA ends was used to search the NCBI database. Once the putative start and stop codons were determined, two specific primers containing start and stop codons were designed to obtain the full-length genes. The gene names *CoD6* and *CtD6* were given to the full-length desaturase genes obtained from *C. obscurus* and *C. thromboides*, respectively.

4.3.5 Cloning of the putative desaturase genes for functional analysis

The primers LT58 and LT59 for *CoD6* and LT37 and LT41 for *CtD6*, respectively, were used to amplify coding regions using the proof reading DNA polymerase, *Phusion* (New England Biolabs, Pickering, Ontario). The fragments were then ligated into the vector pYES2.1/V5-His-TOPO (Invitrogen Canada Inc.) (Figure 4.2) to yield plasmids pLT11 for *CoD6*; pLT2 and pLT3 for *CtD6.* The sequence information of the inserts confirmed they were identical to the original cDNAs and are "in sense" orientation relative to the *GAL1* promoter. The recombinant plasmids and an empty pYES2.1 vector, used as a negative control, were then introduced into the yeast host *S. cerevisiae* INVSc1 (Invitrogen Canada Inc., Burlington, Ontario) by lithium acetate transformation method (Gietz *et al*., 1992). The yeast transformants were selected on uracil-deficient medium as the pYES2.1 expression utilizes uracil as a selection marker.

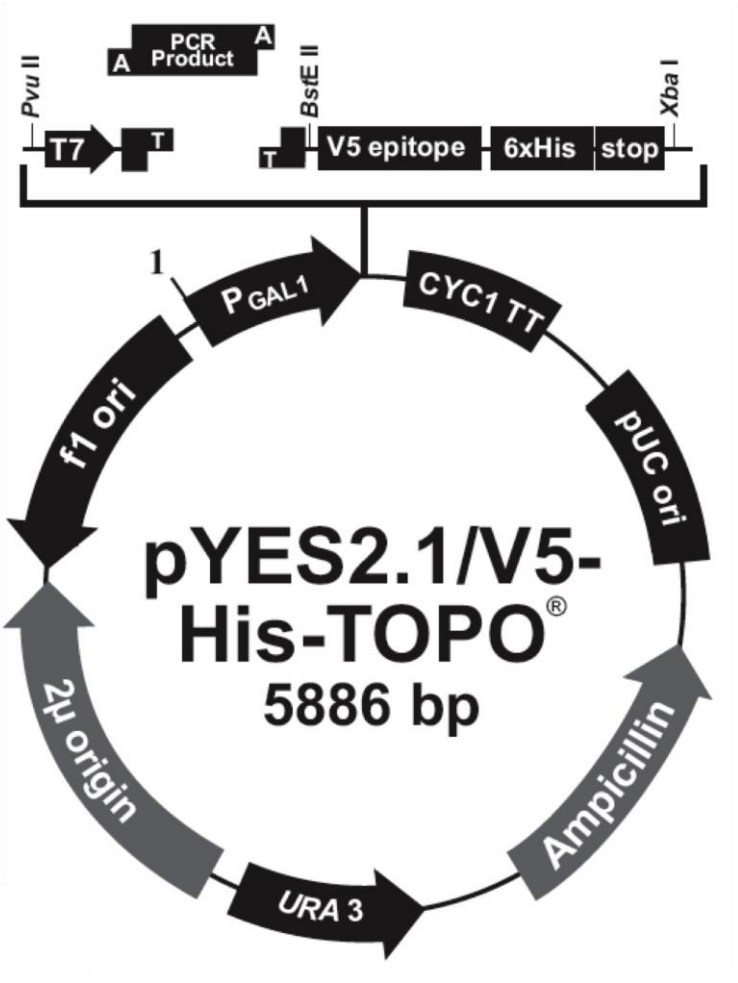


Figure 4.2 The pYES2.1 vector map, this expression system was used for the functional analysis of putative desaturase and elongase genes cloned from *C. obscurus* and *C. thromboides.*

Table 4.1 Primers used in the cloning of Δ6 desaturase from the *Conidiobolus spp.*

Primer Name	Sequence (5' > 3')	Purpose
LT11	TGGTGGAARRAYAANCAYAA	Degenerate Primer
LT14	YTGNARNCCNCCRRGRAACCA	Degenerate Primer
LT16	ATHGMNRANCAYCCNGGNGG	Degenerate Primer
LT23	TCGATTGAGGTACGAAGTTGACG	5'RACE *C. thromboides*
LT25	CAAGTCTAGGCATTGGTGAAGGACG	5'RACE *C. thromboides*
LT29	GTTGAAGATCTGGATCATGTTCTGGGTC	5'RACE *C. thromboides*
LT30	GAGGATATGGCGGGACCATAAAAGC	5'RACE *C. thromboides*
LT50	AGAGTTCCATAGCGTTCTCGGACCAGGC	5' RACE *C. obscurus*
LT51	TGCCATCCACATTGTTGAAAGAAGAGTCC	5' RACE *C. obscurus*
LT22	CCAGAACATGATCCAGATCTTCAGC	3'RACE *C. thromboides*
LT24	CGTCCTTCACCAATGCCTAGACTTG	3'RACE *C. thromboides*
LT52	TGGGTTGGGGGTCACTTCTTTGGAGC	3' RACE *C. obscurus*
LT53	ATCTTGGTGCGCATATAGCATGTGGTTC	3' RACE *C. obscurus*
AP1	CCATCCTAATACGACTCACTATAGGGC	AP1 Adapter Primer
AP2	ACTCACTATAGGGCTCGAGCGGC	AP2 Adapter Primer
LT37	**GCGGATCC**ATTATGGCACCACCACAAGTAATTC ***(BamHI)***	Full length - *C. thromboides* (5' end)
LT41	**GCTAGC**TTAATGAATGGTGTCCATGTTGACTAG ***(NheI)***	Full length - *C. thromboides* (3' end)
LT58	**GGATCC**ATCATGGCACCTCTTACTAAC ***(BamHI)***	Full length - *C. obscurus* (5' end)
LT59	**GGATCC**TTAATCCTGTTTAGGAGGTTCAG ***(BamHI)***	Full length - *C. obscurus* (3' end)

Degenerate nucleotide **Y:** C or T; **R:** A or G; **H:** A, T or C; **N:** A, C, G or T.

All primers were synthesized by Sigma-Aldrich Canada Ltd. Oakville, Ontario.

4.3.6 Heterologous expression of the putative desaturase genes in *S. cerevisiae*

To assess the desaturase activity, the yeast transformants were grown to saturation in 10 mL of synthetic yeast media containing 2% glucose, 0.67% bacto-yeast nitrogen base lacking uracil for 2 days at 28°C. The cultures were then washed twice with distilled water and induced with 10 mL of the induction medium (synthetic yeast media containing 2% galactose) supplemented with or without 250 μM fatty acid substrate in the presence of 0.1% tergitol. The induced cultures were incubated at 20°C for 2 days to allow for adequate gene expression and the synthesis of new products.

4.3.7 Fatty acid analysis

Following induction, the yeast cells were harvested by centrifugation at 2400 rpm and washed once with 15 mL of 0.1% Tergitol and twice with 10 mL of distilled water. The total fatty acid methyl esters (FAME) were prepared directly from washed yeast pellets with 3N methanolic HCl (Meesapyodsuk *et al*., 2007). The FAMEs samples were analyzed on an Agilent 6890N gas chromatograph equipped with a DB-23 column (30 m-0.25-mm) with 0.25-µm-film thickness (J&W Scientific). The column temperature was maintained at 160 °C for 1 min, then raised to 240 °C at a rate of 4 °C/min. (Reed *et al*., 2000)

To determine the substrate specificity of the novel desaturase genes, different fatty acids varying in chain length and double bond position were fed exogenously to the yeast transformants at a final concentration of 250 µM. The fatty acid desaturase products were then analyzed by GC and GC/MS. GC-MS analysis was accomplished using an Agilent 5973 mass selective detector coupled to an Agilent 6890N gas chromatograph using the same column and conditions as described above. The mass selective detector was run by standard electron impact conditions (70 eV), scanning an effective *m*/*z* range of 40–700 at 2.26 scans/s.

4.4 Results

4.4.1 Degenerate RT-PCR amplification of partial cDNA fragments of the putative Δ6 desaturase genes

Various conditions including different concentrations of $MgCl_2$, templates and primers as well as different PCR protocols were attempted with each set of primers in degenerate RT-PCR reactions. In the end the degenerate RT-PCR with the primer set LT11 and LT14 was found to be able to generate a clear band with the expected size of ~500 bp with the RNA of *C. thromboides* (Figure 4.2), whereas the RT-PCR with primer set LT14 and LT16 generated a distinct 1 kb fragment using the RNA from *C. obscurus* (Figure 4.3).

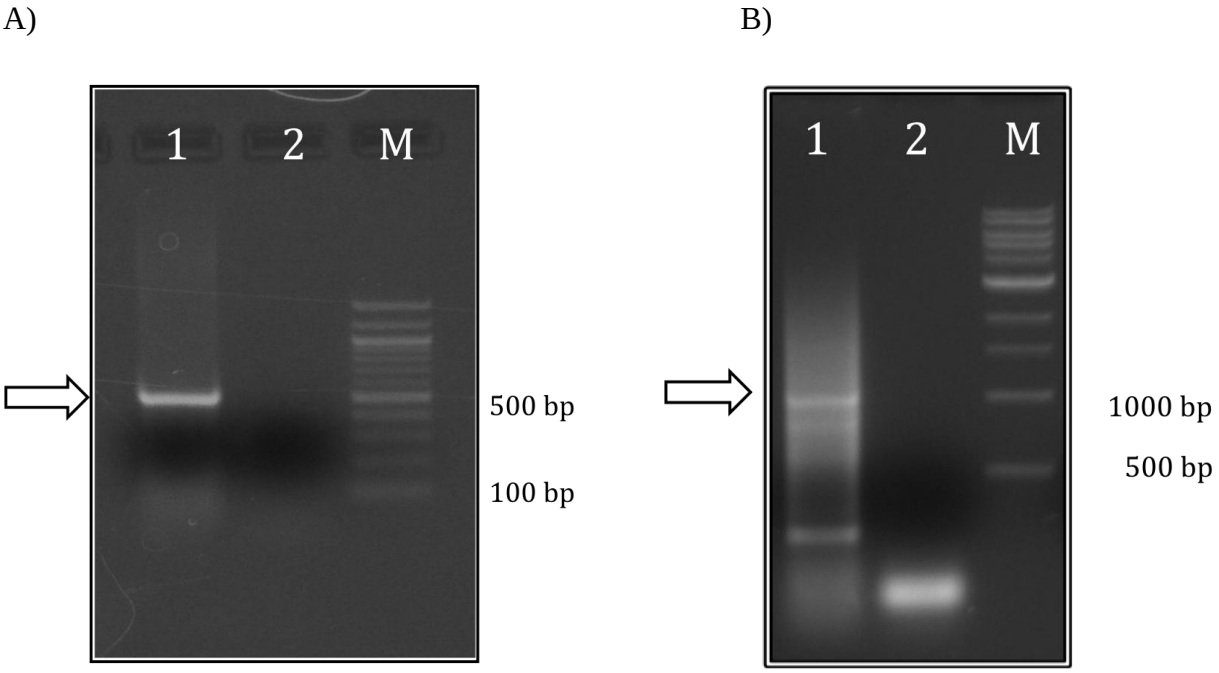


Figure 4.3 Degenerate RT-PCRs with the RNAs isolated from *C. thromboides* (A) and *C. obscurus* (B). **PCR Conditions:** 94°C for 2 minutes, 10 cycles of 94°C for 30 seconds, 50°C for 1 minute, 72°C for 1 minute, 25 cycles of 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, and 72°C for 10 minutes. **1,** PCR with RNAs from *C. thromboides* or *C. obscurus* as the template; **2,** Negative control (without template). **M,** 100 bp ladder marker.

The expected PCR products were gel purified and cloned into the pGEM-T Easy TA cloning vector and transformed into TOP 10F' electrocompetent cells. Twelve transformants were randomly selected from each fungus for sequencing. The sequence information was used to search against the NCBI genbank database to check the homology to other known sequences. The results showed both fragments from *C. thromboides* and *C. obscurus* shared sequence similarity to numerous fatty acid desaturase enzymes in the database, with the top BLAST results resembling Δ6 desaturases from *Mucor rouxi* (70%) and *Mortierella alpina* (60%), respectively. Such a high homology at the amino acid level between the known fungal desaturases and the partial cDNA fragments from the *Conidiobolus spp.* gave strong indication that the RT-PCR with the degenerate primers was effective to isolate partial fragments of the Δ6 desaturase genes from the two fungal species.

In reference to other known Δ6 desaturase enzymes, it was expected that the open reading frame of the desaturase genes from *Conidiobolus spp.* should be ~ 1450 bp in length. Thus, it was expected at least 450 bp at the 5' end and 285 bp at the 3' end of the desaturase gene from *C. thromboides,* and at least 114 bp at the 5' end and 285 bp at the 3' end of the gene from *C. obscurus* were to be determined.

4.4.2 Rapid Amplification of the cDNA Ends (RACE) of desaturases

To retrieve the missing 5' and 3' ends of the putative Δ6 desaturase genes, Rapid Amplification of the cDNA Ends (RACE) was adopted by using Clonetech's Marathon cDNA amplification kit following the manufacturer's recommendations. Gene specific primers were designed based on sequence information of the partial cDNA fragments. RACE amplifications were performed in a series of two PCR reactions. The initial PCR reaction is used to amplify a larger fragment of the gene thereby creating a longer PCR product. This is then followed by the second PCR reaction (nested PCR reaction) using nested primers that are located within the initially amplified product.

The initial 5' RACE PCRs were performed using various combinations of reverse primers and the forward adaptor primer (AP1), which generated faint bands of the

A) B)

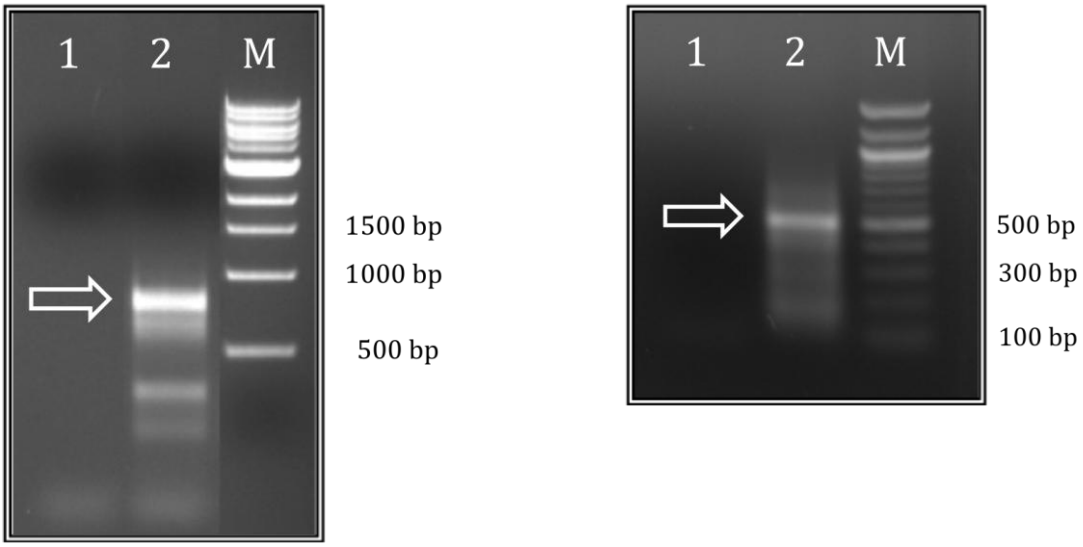


Figure 4.4 Results from the nested 5' RACE for retrieving the 5' ends of putative Δ6 desaturase genes. **A)** ***C. thromboides***: **1,** Negative control **2,** Nested PCR reaction with LT29 and AP2 (853 bp) and **M,** the 1000 bp ladder marker. **PCR conditions**: 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 68°C for 2 minutes. **B)** ***C. obscurus***: **1,** Negative control **2,** Nested PCR reaction with LT51 and AP2 (516 bp) and **M,** 100 bp ladder marker. **PCR conditions**: 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 1 minute.

expected size from *C. thromboides* and *C. obscurus*, respectively. The nested PCRs were then performed using initial RACE products as the template, which produced PCR products of 853 bp from *C. thromboides* and 516 bp from *C. obscurus* **(**Figure 4.4). Both these fragments were then gel-purified and ligated into the sequencing vector, pGEM T Easy, and sequenced. The sequencing results from *C. thromboides* revealed a possible ~72 bp fragment was still missing from the 5' putative start codon. To obtain the missing part, two new 5'RACE primers were designed closer to the cDNA end. The newly-amplified cDNA product from 5'RACE reaction were cloned and sequenced. The results showed the amplified fragment indeed contains the missing region including the putative start codon and about 233 bps of the untranslated region.

Similar to initial 5'RACE result from *C. thromboides*, the 5' RACE product from *C. obscurus* was about 105 bp shorter than the expected size from the 5' start codon. Thus, the same strategy was applied for cloning the 5' missing region of the Δ6 desaturase gene form *C. obscurus*. With new RACE primers, the missing fragment containing the putative start codon and 102 bp of the untranslated region was successfully amplified.

The initial 3' RACE did not generate clear bands with the expected sizes. Nevertheless, the regions with the expect size were still cut, gel-purified and used as templates for the nested 3' RACE. As shown in Figure 4.5, the expected PCR products from 3'RACE reactions were amplified by the nested PCR using first PCR products as templates. Both 3' fragments of the desaturase genes from *C. thromboides* and *C. obscurus* were gel-purified and cloned into pGEM–T EASY vector for sequence analysis. The sequencing results indicated both the 3' ends of the desaturase genes from *C. thromboides and C. obscurus* were successfully isolated.

4.4.3 Cloning of the full-length desaturase genes from *C. thromboides* and *C. obscurus*

With 5' and 3' RACE, missing ends of both putative Δ6 desaturase cDNAs from *C. thromboides and C. obscurus* were finally retrieved. Assembly of theses fragments revealed that the full-length cDNA of the Δ6 desaturase from *C. thromboides* (*CtD6*) was

A) B)

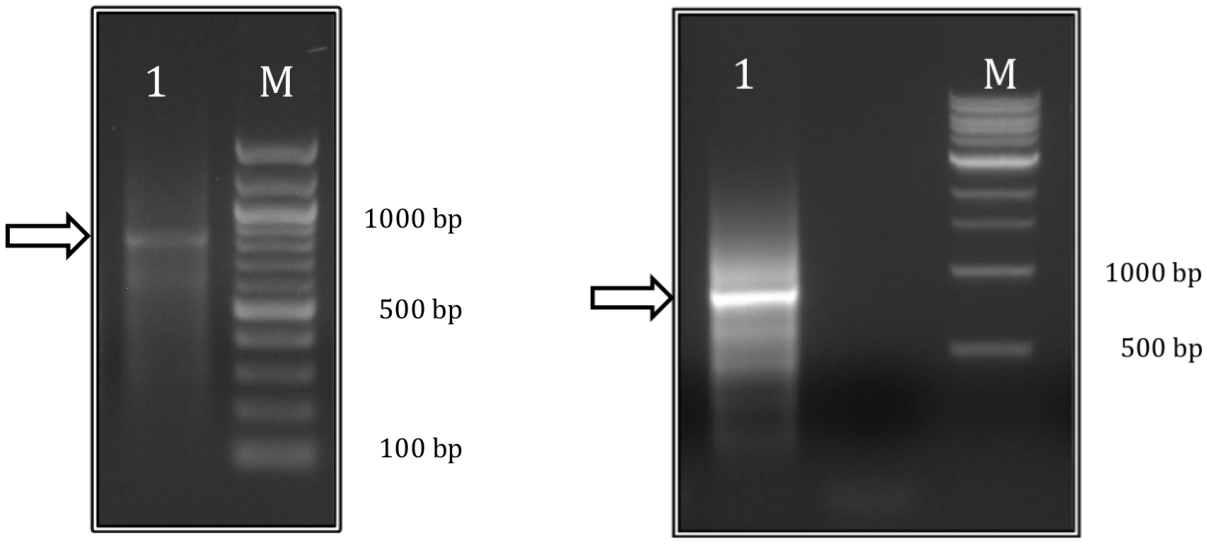


Figure 4.5 Results from the 3' nested RACE for retrieving the 3' ends of the putative Δ6 desaturase genes. **A)** ***C. thromboides****:* **1,** Nested PCR with primers LT24 and AP2. **M:** 100 bp ladder marker. PCR conditions: 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 68°C for 2 minutes **B)** ***C. obscurus***: **1,** Nested PCR with LT53 and AP2. **M,** 1000 bp ladder marker. The PCR conditions used for this reaction were 94**°**C for 2 minutes, 35 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 1 minute.

1713 bp in length, whereas the full-length cDNA of the Δ6 desaturase from *C. obscurus* (*CoD6*) was 1614 bp long (Figure 4.6 and 4.7).

The identification of the putative start codons of *CtD6* and *CoD6* open reading frames was based on the presence of an in-frame stop codon upstream of the initiation site in the full-length cDNAs (Figure 4.6 and 4.7). For *CtD6*, the stop codon was found almost adjacent to the start site, whereas for CoD6 the stop codon was found 100 bp upsteam from the start site. In both cases the start site was confirmed through multiple sequence alignments.

The two full-length Δ6 desaturase cDNAs including putative start and stop codons from *C. thromboides* and *C. obscurus* were then amplified separately by RT-PCR using specific primers. The amplified fragments were subsequently cloned into a vector and sequenced.

4.4.4 Sequence analysis of the putative Δ6 desaturases from the *Conidiobolus spp*.

The sequencing results of the full-length *CtD6* cDNAs retrieved by the specific RT-PCR indicated the presence of two different isomers for the putative Δ6 desaturase gene from *C. thromboides*. Sixty-three nucleotide substitutions were found throughout the sequences of the two isomers, of which thirteen in the open reading frame resulted in amino acid changes. Initially, it was believed this might be due to PCR error, thus additional clones were analyzed through sequencing. However, the sequencing results confirmed there are two different cDNA isomers for the desaturase gene. Both *CtD6* isomers contain an opening reading frame (ORF) of 1,486 bp in length encoding a polypeptide of 467 amino acids with a molecular weight of 54.4 kDa (Figure 4.5). The start codon of both isomers was located at 233 bp from the 5' end, and the stop codon resided at 1,649 nucleotides downstream of the start site, giving 233 bp long at the 5'untranslated region (UTR) and 64 bp at the 3' UTR.

Sequencing results of the full-length *CoD6* cDNAs retrieved by specific RT-PCR indicated there was only one cDNA species for the putative Δ6 desaturase gene from *C. obscurus*. The *CoD6* gene contains an ORF of 1,362 bp with the start codon located at 161 bp from the 5' end, and the stop codon located at 96 bp from the 3' end. The ORF of *CoD6* encodes a polypeptide of 449 amino acids in length with a molecular weight of 51.7 kDa (Figure 4.7).

The hydropathy plot analysis of the two deduced amino acid sequences CtD6 and CoD6 revealed two hydrophobic regions in both sequences that are typical features of this type of membrane desaturases (Figure 4.8). The first hydrophobic region was located between the heme binding site and the histidine rich motif I (from about 100 amino acid to 150 amino acid), while the second hydrophobic region was found between histidine motif II and III (from about 250 animo acid to 350 amino acid). These hydrophobic regions were also observed in other membrane bound Δ6 desaturases from the fungus *Mortierella alpina* and *Mucor rouxii*, and plant *Borago officinalis* and *Physcomitrella patens* (Laoteng *et al*., 2000).

The sequence comparison indicated the two putative Δ6 desaturases CtD6 and CoD6 share 30% amino acid identity. The comparison of the two deduced polypeptides with other related sequences showed that CtD6 has high amino acid identity to fungal desaturases from *Aspergillus fumigatus* (42%), *Rhizomucor pusillus* (36%), *Mucor rouxii* (36%) and *Mucor circinelloides* (35%), whereas CoD6 has high sequence identity to fungal Δ6 desaturases from *Mortierella alpina* (47%), *Mortierella isabellina* (46%), *Rhizopus stolonifer* (42%), *Mucor circinelloides* (40%) and *Thamnidium elegans* (38%).

Alignment of the two putative Δ6 desaturases with related enzymes revealed that three conserved histidine-rich motifs for di-iron binding at the catalytic center and a cytochrome b5-like domain including the heme-binding motif, H-P-G-G, were highly conserved among these sequences. In comparison with the previously identified fungal Δ6 desaturases, the heme binding site and three conserved histidine boxes all appeared to be similarly spaced between each other (Figure 4.9).

Phylogenetic analysis showed that CoD6 and CtD6 were clustered into two separate groups. CoD6 more resembles fungal Δ6 desaturases, whereas CtD6 is more closely

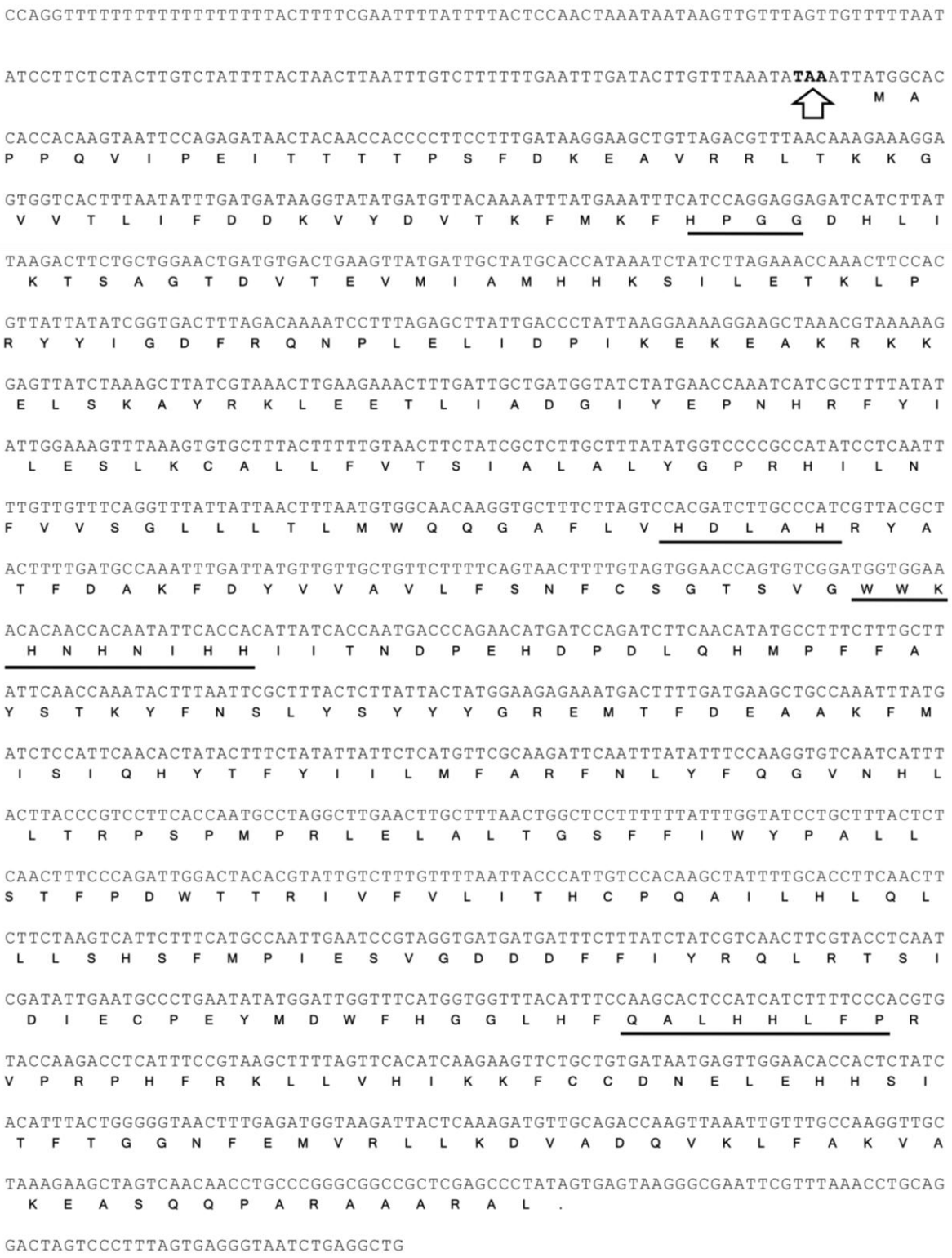


Figure 4.6 The full-length cDNA of *CtD6-1* and the translated ORF. The heme-binding site and three histidine motifs are highlighted. The stop codon in the 5' upstream is indicated with an arrow.

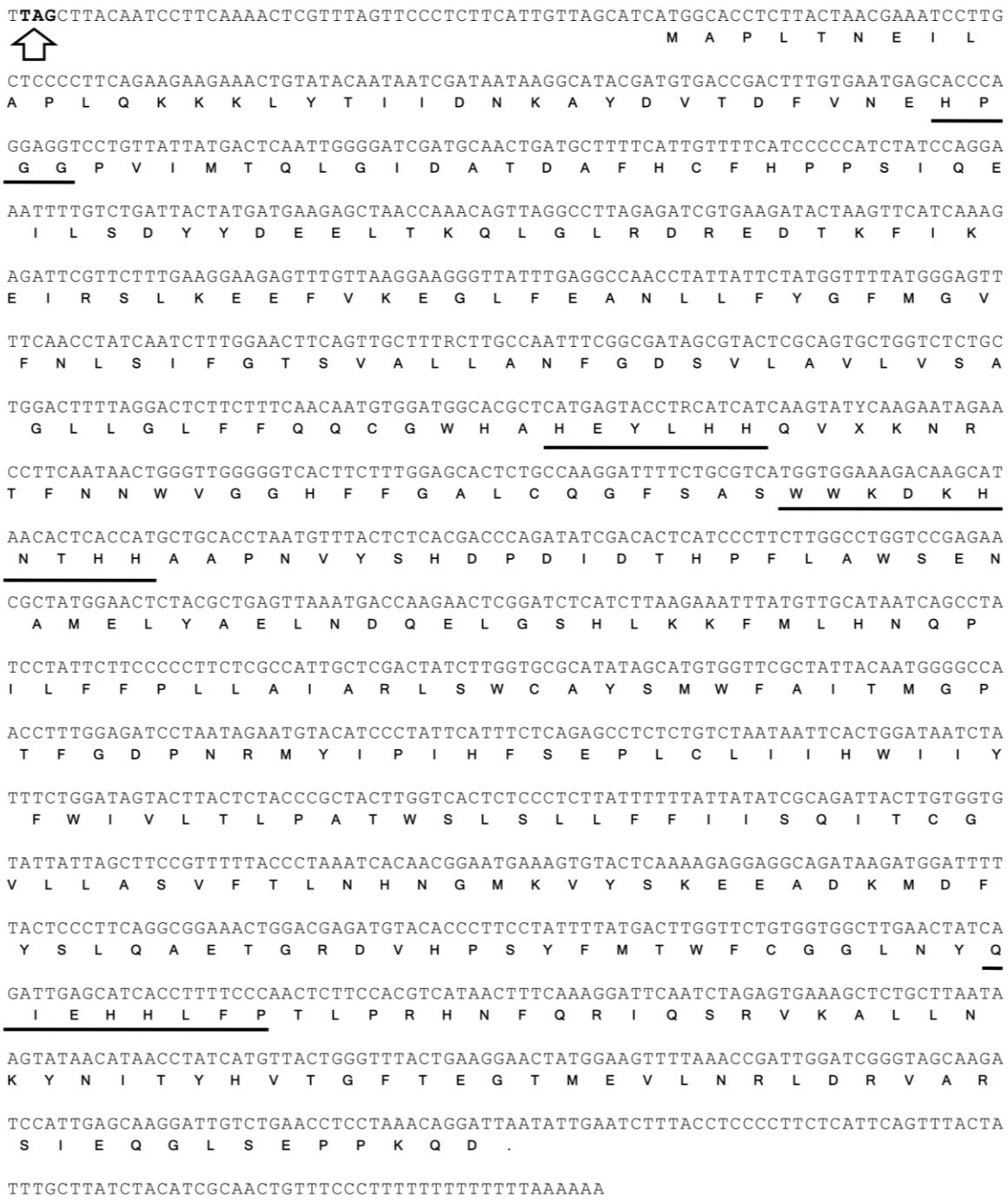
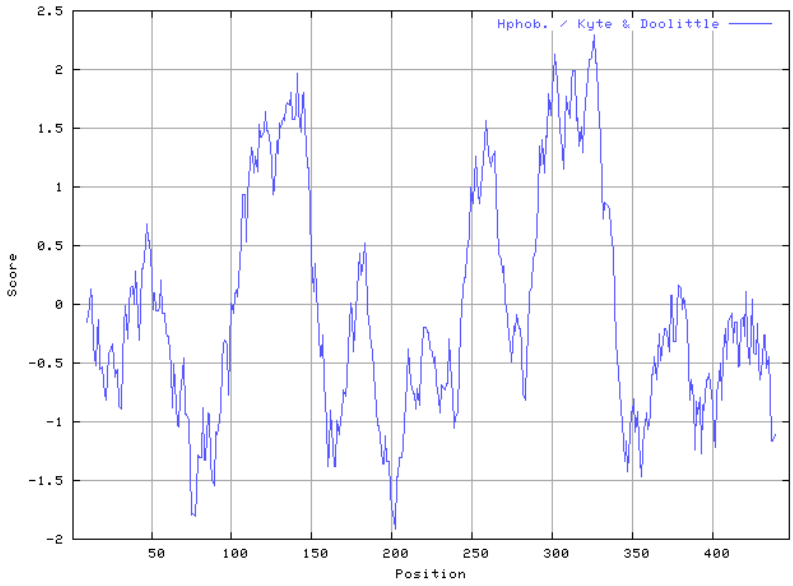


Figure 4.7 The full-length cDNA of *CoD6* and the translated ORF. The heme-binding site and three histidine motifs are highlighted. The stop codon in the 5' upstream is indicated with an arrow.

A)



B)

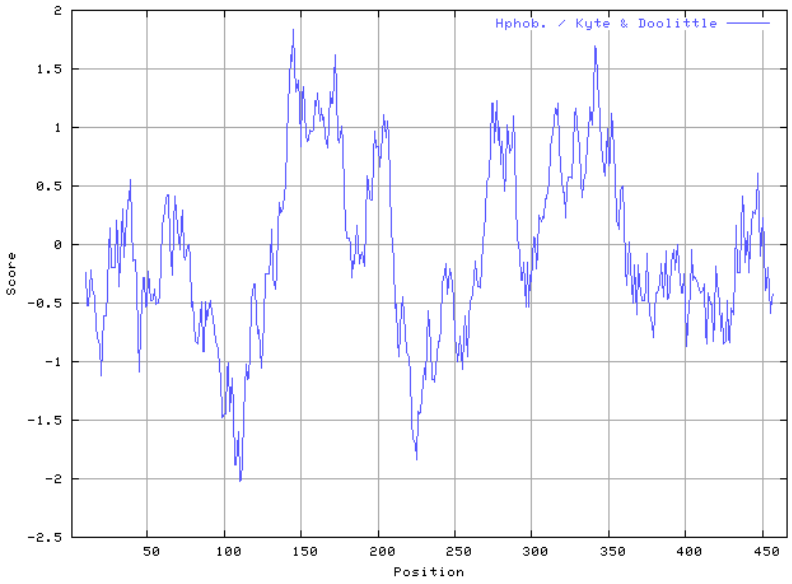


Figure 4.8 Kyte-Doolittle hydropathy plots of CoD6 (A) and CtD6 (B). Protscale was used to determine the hydrophobic profile of the polypeptide, based on Kyte & Doolittle parameters (Kyte and Doolittle, 1982). Window size, 19. Peaks greater than 1.6 in vertical axes indicate the presence of hydrophobic domains.

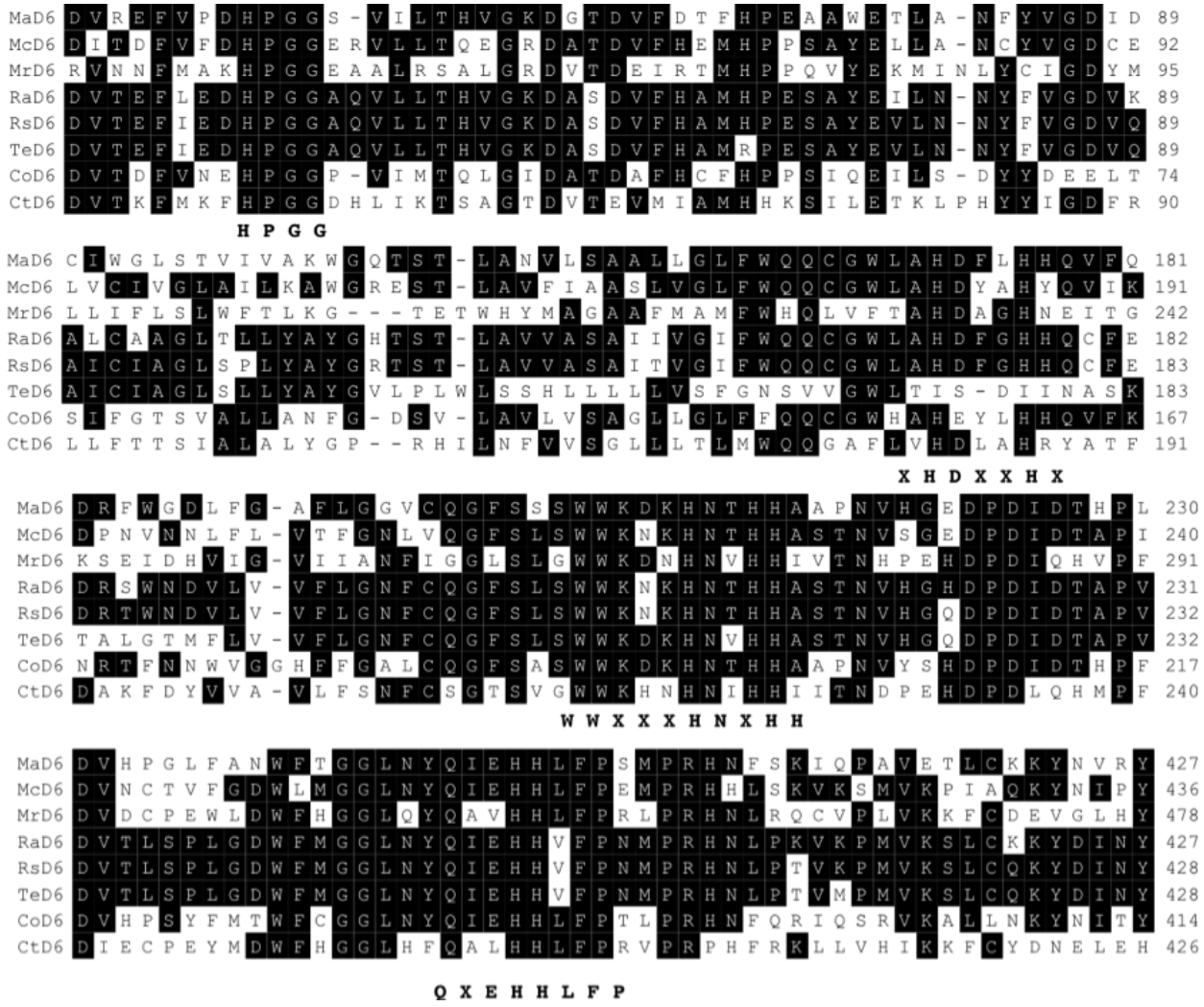


Figure 4.9 Alignment of two putative Δ6 desaturases with related protein sequences including *Mortierella alpina* (**MaD6**, AAF08685), *Mucor circinelloides* (**McD6**, BAB69055), *Rhizophus arrhizus* (**RaD6**, AAS93682), *Rhizopus stolonifer* (**RsD6** ABB96724), *Mucor rouxii* (**MrD6D**, AAG36959), *Mucor circinelloides* (**McD6**, BAB69055), *Thamnidium elegans* (**TeD6**, AAX40418), *Conidiobolus obscurus* (**CoD6**), *Conidiobolus thromboides* (**CtD6**). Shown above are the highly conserved regions found among all desaturases.

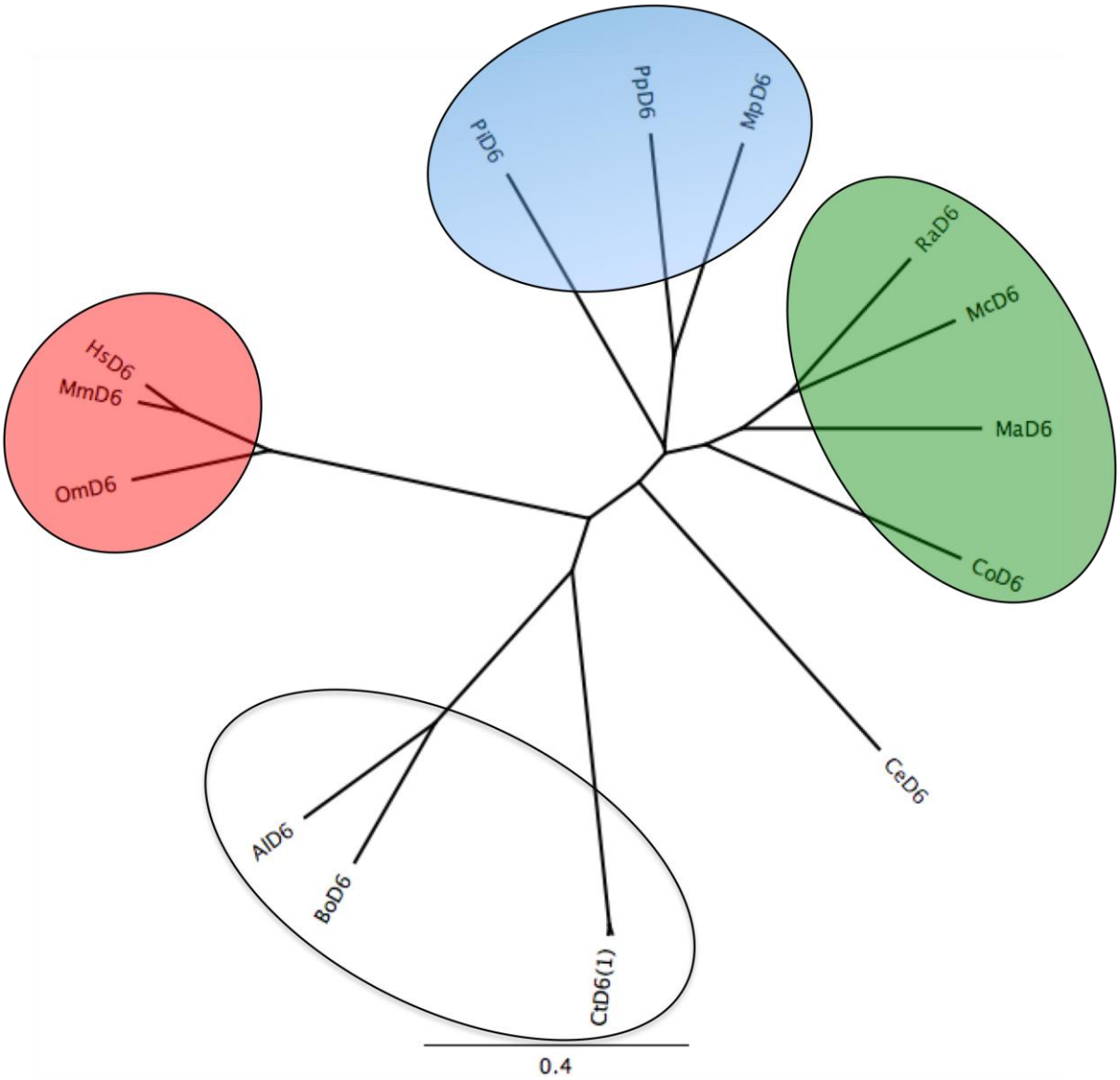


Figure 4.10 Phylogenetic analysis of **CtD6** (1) and **CoD6** with related sequences. The DDBJ/EMBL/GenBank accession numbers of the different protein sequences are as follows: *Anemone leveillei* (**AlD6**, AF536525), *Caenorhabditis elegans* (**CeD6,** AF031477), *Borago officinalis* (**BoD6**, AF007561), *Mucor circinelloides* (**McD6**, AB052086), *Marchantia polymorpha* (**MpD6**, AY583463), *Mortierella alpina* (**MaD6**, AF110510), *Mus musculus* (**MmD6**, AF126798), *Physcomitrella patens* (**PpD6**, AJ222980), *Pythium irregulare* (**PiD6**, AF419296), *Homo sapiens* (**HsD6**, AF084559), *Oncorhynchus mykiss* (**OmD6**, AF301910), *Rhizopus arrhizus* (**RaD6**, AAP83964). The unrooted phylogram was constructed by the UPGMA method using Geneious 4.70.

related to plant Δ6 desaturases (Figure 4.10).

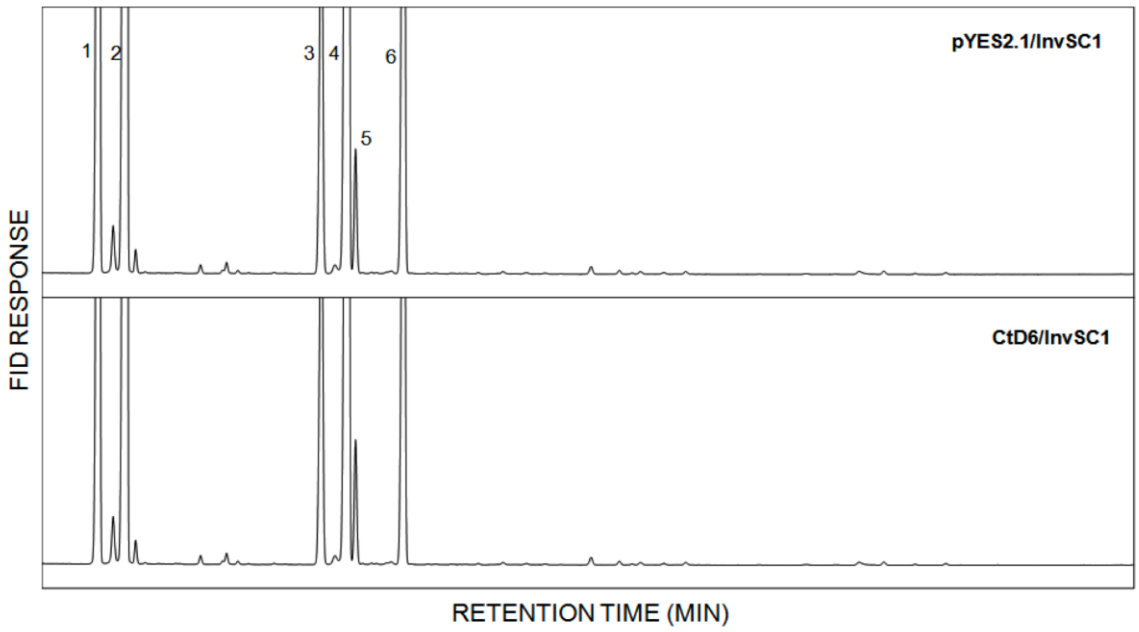
4.4.5 Functional expression of *CtD6* and *CoD6* in yeast

To determine the function of *CtD6*, the ORFs of both cDNA isoforms were cloned into a yeast expression vector pYES2.1 under the guidance of *GAL1* promoter. The recombinant plasmids were then transfomed into the yeast *S. cerevisiae* INVSc1. Transformants were selected on a selective medium and screened by PCR using a primer in the promoter and a primer in the transgene.

Selected transformants were grown in the presence of linoleic acid or linolenic acid, two possible substrates for Δ6 desaturase. Fatty acid analysis of transformants expressing *CtD6* indicated both isomers of *CtD6* isolated from *C. thromboides* could not convert LA or ALA, into its respective Δ6 desaturated products, GLA and SDA as expected, and transformants produced fatty acid profiles identical to the control yeast with the empty vector, indicating the two *CtD6* cDNAs did not have any Δ6 desaturase activity under the experimental conditions (Figure 4.11). To determine whether the two *CtD6* cDNAs act as other front-end desaturase such as Δ4 desaturase, Δ5 desaturase or Δ8 desaturase, the two transformants were fed with DPA, DGLA, ETA and 20:2-11,14. Fatty acid analysis showed transformant cultures still produced fatty acid profiles identical to those of the control cultures, indicating these two *CtD6* cDNA isomers did not possess any Δ4 desaturase, Δ5 desaturase or Δ8 desaturase activity.

To define the function of *CoD6*, the ORF was cloned into a yeast expression vector pYES2.1 under the guidance of *GAL1* promoter. The plasmid was then transformed into *S. cerevisiae* INVSc1. Fatty acid analysis showed that compared with the control, transformants expressing *CoD6* in presence of linoleic acid produced a new peak with the retention time identical to that of GLA. GC/MS analysis of this peak confirmed it is GLA (Figure 4.12), indicating *CoD6* isolated from *C. obscurus* codes for a functional Δ6 desaturase which could introduce a Δ6 double bond into linoleic acid.

A)



B)

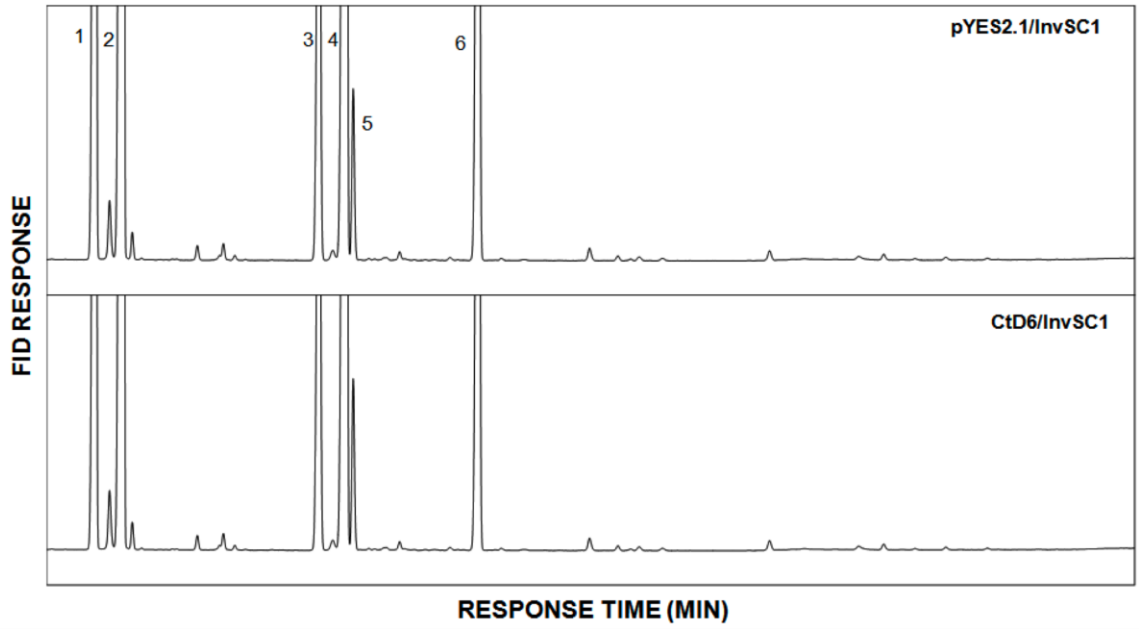


Figure 4.11 GC analysis of FAMEs prepared from transformants expressing *CtD6* in presence of linoleic acid (A) and α-linolenic acid (B). **Fatty acid peaks:** (1) 16:0, (2) 16:1-9, (3) 18:0, (4) 18:1-9, (5) 18:1-11, (6) 18:2-9,12 (A) or 18:3-9,12,15 (B)

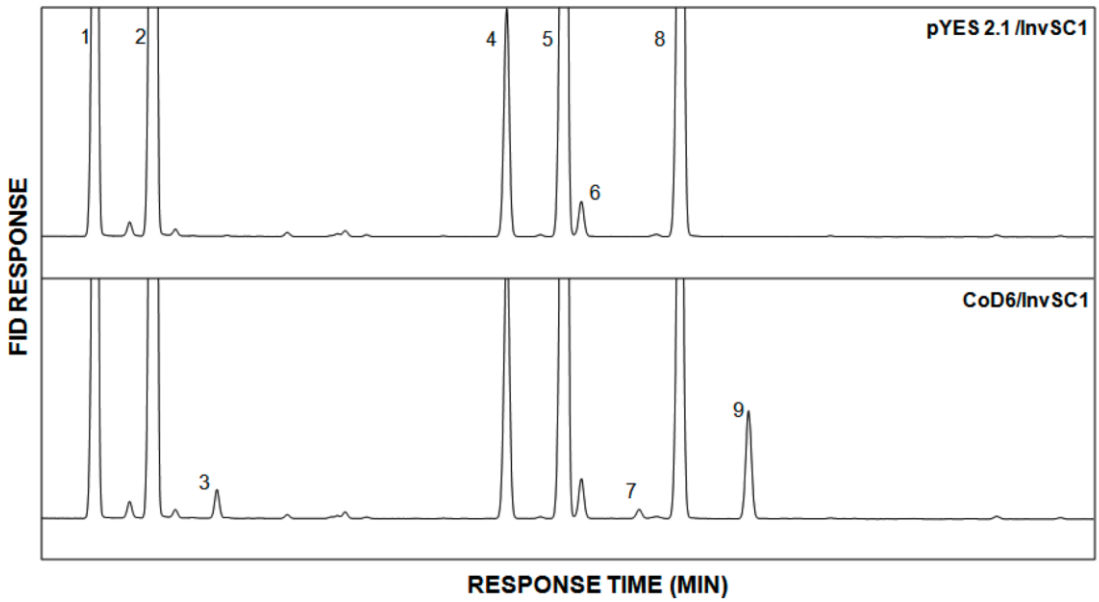


Figure 4.12 Functional analysis of *CoD6* with linoleic acid (18:2-9,12). **Fatty acid peaks:** (1) 16:0, (2) 16:1-9, (3) 16:1-6,9, (4)18:0, (5) 18:1-9, (6) 18:1-11, (7) 18:2-6,9, (8) 18:2-9,12 and (9) 18:3-6,9,12.

To study the substrate specificity of *CoD6*, transformants were supplied with several other fatty acids that differed in number and position of double bonds as well as in chain length. The results from these feeding experiments showed that the highest desaturation activity was detected with LA and ALA and similar conversion efficiency was observed with these two substrate (15% and 15.7% respectively). Besides introducing double bonds into LA and ALA, CoD6 could also, to a lesser extent, use 16:1-9 and 18:1-9 as substrates, producing 16:2-6,9 and 18:2-6,9, respectively. Whereas, 17:1-10, 19:1-10 and 20:2-11,14 were not able to act as substrates for CoD6 (Table 4.2).

Table 4.2 The conversion efficiency of substrates in the yeast strain CoD6/INVSc1 expressing *C. obscurus* Δ6 desaturase.

Substrate		**Product**		**Conversion Efficiency**
Fatty Acid	**%TFA**	**Fatty Acid**	**%TFA**	
16:1-9	46.88±1.28	16:2-6,9	1.39±0.24	2.89±0.49
18:1-9	27.27±0.99	18:2-6,9	0.44±0.09	1.58±0.33
18:2-9,12	11.70±0.14	18:3-6,9,12	2.07±0.02	15.06±0.18
18:3-9,12,15	13.26±0.09	18:4-6,9,12,15	2.48±0.10	15.76±0.46

Note: Values represented as the weight percentage of the total fatty acids (TFA%)

4.5 Discussion

In this study, two cDNA sequences coding for putative Δ6 desaturase were isolated by degenerate RT-PCR and RACE from *C. obscurus* and *C. thromboides*. The full length cDNA from *C. thromboides* (*CtD6*) contained an opening reading frame (ORF) of 1,416 bp encoding a polypeptide of 466 amino acids, while the full length cDNA from *C. obscurus* (*CoD6*) contained an ORF of 1,357 bp encoding a polypeptide of 447 amino acids. To determine the function of the putative desaturase genes, the two cDNAs were expressed separately in the yeast INVSc1 under a galactose-inducing promoter. The expression showed *CoD6* encoded a functional Δ6 desaturase that can introduce a double bond at the sixth position of linoleic acid and α-linolenic acid, whereas *CtD6* does not exhibits any Δ6 desaturase activity when expressed in yeast. In addition, although CoD6 was functional in yeast, the desaturase activity was relatively low in comparison to other previously characterized Δ6 desaturases from fungi. The Δ6 desaturated products of two preferred substrates, linoleic acid and linolenic acid were in a range of 2 to 3% compared to 10% of *M. alpina* Δ6 desaturase (Huang *et al*., 1999), 7.1% of *M. rouxii* Δ6 desaturase (Laoteng *et al*., 2000), 6.0% of *P. irregulare* Δ6 desaturase (Hong *et al*., 2002) and 4.5% of *Rhizopus arrizhus* Δ6 desaturase (Zhang *et al*., 2004).

It is unexpected that CtD6 cloned from *C. thromboides* does not have Δ6 desaturase activity. CtD6 shares 30% amino acid identity with CoD6 and high amino acid identity to other fungal Δ6 desaturases from *Aspergillus fumigatus* (42%), *Rhizomucor pusillus* (36%), *Mucor rouxii* (36%) and *Mucor circinelloides* (35%). The full alignment of the amino acid sequences with previously cloned Δ6 desaturases shows that like all other Δ6 desaturases, CtD6 possesses three typical histidine-rich motifs and a heme-binding domain that are believed to be involved in the catalysis of the desaturation reaction. However, a close look at these conservative regions revealed that the residues surrounding the first histidine box (A-F-L-V-H-D) and the third histidine box (Q-A-L-H-H-L-F-P) of CtD6 were not completely conserved compared with those (G-W-X-X-H-D in the first histidine box and the last histidine box Q-I-E-H-H-L-F-P) of functionally characterized Δ6 desaturases from other organisms including the Δ6 desaturase CoD6 from *C. obscurus*. CtD6 has two amino acid replacements (A-F vs G-W) in the first histidine box and two amino acid substitutions (A-L vs I-E) in the third histidine box (Fig. 4.13 and Fig. 4.14). Previous studies have shown that amino acids in the regions of histidine boxes are critical for the activity of the

Δ6 desaturases, mutations at the conserved leucine and glutamine residue in the boxes were shown to reduce the enzymatic activity by 10% (Sayanova *et al*., 2004). Thus, the amino acid substitutions in the two regions of CtD6 collectively may have severe impact on the function of the cloned enzyme.

Another possibility is the substrate requirement of *CtD6* might be different from that of conventional Δ6 desaturases and under our assay conditions, this requirement could not be met properly. For instance, Δ6 desaturase and Δ8-sphingolipid desaturase share similar primary structure, but their substrate requirement is distinctly different. The former uses phospholipids or acyl-CoA as substrates, whereas the later utilizes sphingolipids as substrates (Garcia-Maroto *et al*., 2007). However, whether *CtD6* has sphingolipid desaturase activity still remains to be determined.

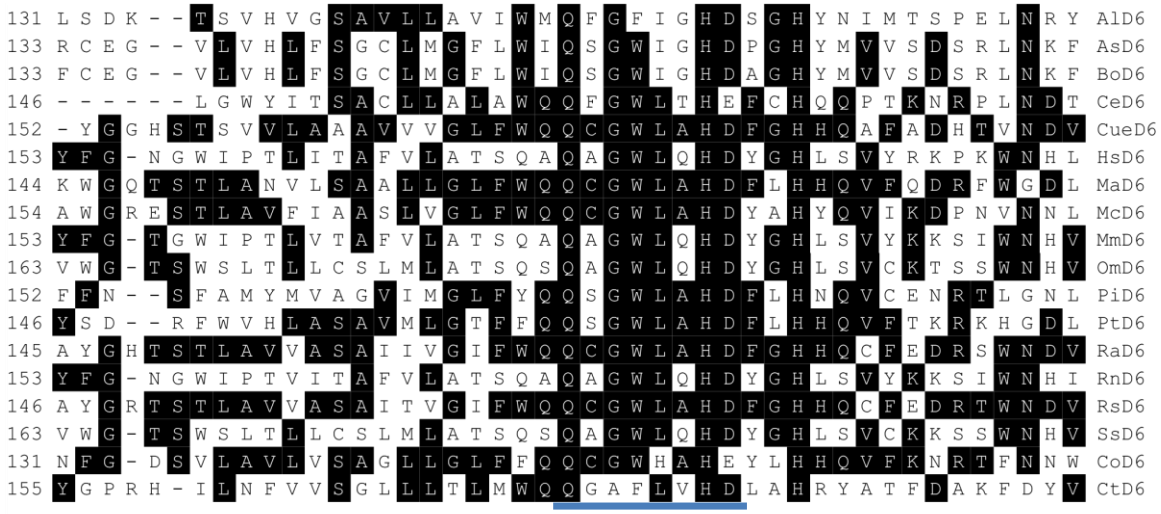


Figure 4.13 Amino acid alignment of the first histidine box of related Δ6 desaturases. Highlighted in black are the highly conserved amino acid residues. Underlined are the conserved residues adjacent to the first histidine box. *Anemone leveillei* (**AlD6**, AAQ10731), *Argania spinosa* (**AsD6,** AAM94345), *Borago officinalis* (**BoD6**, AAD01410), *Caenorhabditis elegans* (**CeD6**, AAC15586), *Cunninghamella echinulata* (**CueD6**, ABA06503), *Homo sapiens* (**HsD6**, AAD20018), *Mortierella alpina* (**MaD6**, AAF08685), *Mucor circinelloides* (**McD6**, BAB69055), *Mus musculus* (**MmD6**, AAD20017), *Oncorhynchus mykiss* (**OmD6**, NP_001117759), *Pythium irregulare* (**PiD6**, AAL13310), *Phaeodactylum tricornutum* **(PtD6**, XP_002182901), *Rhizopus arrhizus* (RaD6, AAP83964), *Rattus norvegicus* (**RnD6**, BAA75496), *Rhizopus stolonifer* (**RsD6**, ABB96724), *Salmo salar* (**SsD6**, NP_001117047), *Thamnidium elegans* (**TeD6**, AAX40418), *Conidiobolus obscurus* (**CoD6**), *Conidiobolus thromboides* (**CtD6**). Alignment was constructed with DNAstar sequence analysis software.

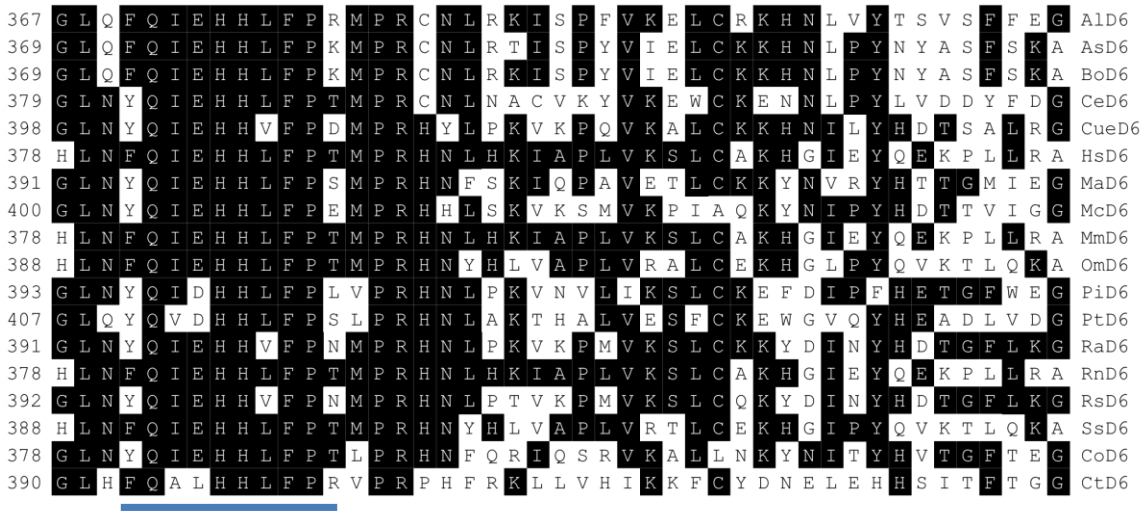


Figure 4.14 Amino acid alignment of the third histidine box of related Δ6 desaturases. Highlighted in black are the highly conserved amino acid residues. Underlined are the conserved residues in the third histidine box. *Anemone leveillei* (**AlD6**, AAQ10731), *Argania spinosa* (**AsD6,** AAM94345), *Borago officinalis* (**BoD6**, AAD01410), *Caenorhabditis elegans* (**CeD6**, AAC15586), *Cunninghamella echinulata* (**CueD6**, ABA06503), *Homo sapiens* (**HsD6**, AAD20018), *Mortierella alpina* (**MaD6**, AAF08685), *Mucor circinelloides* (**McD6**, BAB69055), *Mus musculus* (**MmD6**, AAD20017), *Oncorhynchus mykiss* (**OmD6**, NP_001117759), *Pythium irregulare* (**PiD6**, AAL13310), *Phaeodactylum tricornutum* **(PtD6**, XP_002182901), *Rhizopus arrhizus* (RaD6, AAP83964), *Rattus norvegicus* (**RnD6**, BAA75496), *Rhizopus stolonifer* (**RsD6**, ABB96724), *Salmo salar* (**SsD6**, NP_001117047), *Thamnidium elegans* (**TeD6**, AAX40418), *Conidiobolus obscurus* (**CoD6**), *Conidiobolus thromboides* (**CtD6**). Alignment was constructed with DNAstar sequence analysis software.

5.0 Study 3: Cloning and functional characterization of genes encoding for Δ6 elongase from *C. thromboides* and *C. obscurus*

5.1 Abstract

The genes encoding putative Δ6 elongases (*CoE6* and *CtE6*) were cloned from *C. thromboides* and *C. obscurus* by the RT-PCR approach. Degenerate primers were designed to target the conserved regions of the Δ6 elongases previously identified from other species. Degenerate RT-PCR amplified a portion of the Δ6 elongase genes from the two fungal species. The full-length genes (*CoE6* and *CtE6*) were obtained by the 5' and 3' RACE method. To functionally characterize the putative elongase genes, the ORFs were cloned into a yeast expression vector, which were then transformed into the yeast host. In the presence of exogenously fed substrates, stearidonic acid and γ-linolenic acid, the transformants containing *CoE6* and *CtE6* produced Δ6 elongated products, dihomo-γ-linolenic acid and eicosatetraenoic acid, indicating that both *CoE6* and *CtE6* code for functional Δ6 elongases that can elongate Δ6 polyunsaturated fatty acids by a two carbon unit.

5.2 Hypothesis

The presence of dihomo-γ-linolenic acid (DGLA) eicosapentaenoic acid (EPA) and arachidonic acid (ARA) in the *Conidiobolus spp.* implies there might exist the corresponding biosynthetic pathway for these polyunsaturated fatty acids (PUFAs). The genes encoding Δ6 elongase in the pathway could thus be isolated by the degenerate RT-PCR approach and function of these genes could then be determined in yeast *Saccharomyces cerevisiae.*

5.3 Experimental Approach

5.3.1 RNA Isolation

RNA isolation followed the protocol in section 4.3.1

5.3.2 Designing of degenerate primers for the isolation of fragments of Δ6 elongase genes from *Conidiobolus spp.*

To design the degenerate primers for cloning of the genes encoding Δ6 elongases from *C. thromboides* and *C. obscurus* by RT-PCR, several Δ6 elongases previously identified from other

species were aligned using the DNAstar program, MegAlign. As shown in Figure 5.1, the shaded regions indicate amino acid sequences that were highly conserved through the many species, and thus could be potential target sites for degenerate primer design.

The designing of degenerate primers for isolation of Δ6 elongase genes was a little more difficult due to the fact there was few sequencing information available pertaining to the fungal elongases. To overcome this shortage of sequence information, a wide array of elongases from a broad range of organisms were selected including those from animals, algae and moss as well as fungal species. In addition, the relatively small size of the elongase gene (~956 bp) and lack of conserved regions presented another obstacle to the design of the degenerate primers. Due to the limitation, only two primers LT5 (Forward Primer) and LT6 (Reverse Primer) were designed for RT-PCR. The forward primer was targeted to the only histidine box in the elongases (F-L-H-V-Y-H-H), while the reverse primer was targeted to the conserved tyrosine box region (M-Y-T-Y-Y-F-L-S) which is only ~50 amino acids away from the histidine box. The combination of these two primers should yield a fragment of ~150 bp.

5.3.3 Degenerate RT-PCR

The degenerate RT-PCRs followed the protocol in section 4.3.3

5.3.4 Rapid amplification of the cDNA ends (RACE)

Multiple gene-specific primers were designed on the sequence data obtained from the partial cDNA fragment of the *Δ*6 elongase genes cloned by RT-PCR. The primers used to obtain the missing 5' and 3' ends of the elongase genes are shown in Table 5.1.

Once the putative full-length genes were obtained by RACE, two new gene-specific primers containing start and stop codons were designed to retrive the full-length genes. The gene names *CoE6* and *CtE6* were given to the full-length elongase genes obtained from *C. obscurus* and *C. thromboides*, respectively.

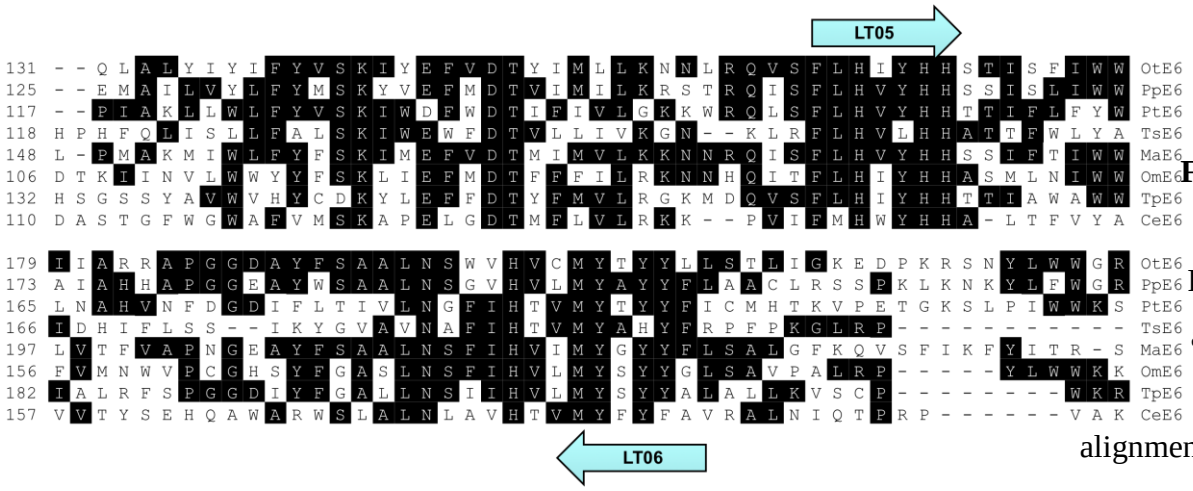


Figure 5.1 Partial amino acid alignment of Δ6 elongases identified from organisms including *Morteriella alpina* (**MaE6,** AAF70417**)**, *Thalassiosira pesudomona* (**TpE6,** AAV67799), *Caenorhabditis elegans* (**CeE6,** CAA92958), *Thraustochytrium spp.* (**TsE6,** CAJ30819), *Physcomitrella patens* (**PpE6,** AAL84174), *Ostreococcus tauri* (**OtE6,** AAV67798), *Phaeodactyluan triconrnutum* (**PtE6,** CAM55851) and *Oncorhynchus mykiss* (**OmE6,** AAV67803). The shaded regions are amino acids that are highly conserved through the different species.

5.3.5 Cloning of the ORFs of putative elongase genes for functional analysis

The primers LT60 and LT61 for *CoE6* and LT39 and LT40 for *CtE6* were used to amplify respective coding regions using the proofreading DNA polymerase, *Phusion.* The fragments were then ligated into the vector pYES2.1/V5-His-TOPO to yield plasmids pLT12 for *CoE6* and pLT1 for *CtE6.* The inserts were confirmed by sequencing to be identical to the original cDNAs and "in sense" orientation relative to the *GAL1* promoter. The recombinant plasmids and an empty pYES2.1 vector used as a negative control were introduced into the yeast host *S. cerevisiae* INVSc1 using the lithium acetate transformation method (Gietz *et al*., 1992). The yeast transformants were selected on a uracil-deficient medium.

5.3.6 Heterologous expression of the putative elongases in *S. cerevisiae*

Heterologous expression of genes followed the same protocol as in section 4.3.6.

5.3.7 Fatty acid analysis

Fatty acid analysis followed the same protocol as in 4.3.7.

Table 5.1 Primers used in the cloning of Δ6 elongases from the *Conidiobolus spp.*

Primer Name	**Sequence**	**Purpose**
LT5	TTYTTNCAYGTNTAYCAYCA	Degenerate Primers
LT6	ARRAARTARTANCCRTACAT	Degenerate primers
LT19	GCAGCTGAGAAATAAGCATCACC	5' RACE *C. thromboides*
LT21	GGTGCCATCAAGGTAACAATCCACC	5' Race *C. thromboides*
LT31	GGCATCCATACGTTAACTTTGGCTTCT	5' Race *C. thromboides*
LT32	CGTGGGAGGAAGCTAGAAGCTACTTTG	5' Race *C. thromboides*
LT56	ATCACGTGGATGTAGGAGTTAAGGGCAG	5' Race *C. obscurus*
LT57	AACCACCAGACGCCAAAGATGGAGCAG	5' Race *C. obscurus*
LT18	GGTGGATTGTTACCTTGATGGCACC	3' end *C. thromboides*
LT20	GGTGACGCTTTATTTCTCAGCCATCACC	3' end *C. thromboides*
LT54	TCTTCCACGTCTACCACCACTGCTCC	3' end *C. obscurus*
LT55	TCAGCTGCCCTTAACTCCTACATCCACG	3' end *C. obscurus*
AP1	CCATCCTAATACGACTCACTATAGGGC	Adapter Primer
AP2	ACTCACTATAGGGCTCGAGCGGC	Adapter Primer
LT39	**GGAATTC**GAAATGAGTTTATTAAATACATTG GATA ***(EcoRI)***	Full length- *C. thromboides* (5' end)
LT40	**GGAATTC**TAATTCATTTTCTTTCCCTTTTG ***(EcoRI)***	Full length- *C. thromboides* (3' end)
LT60	**GCGGCCGC** ATTATGGCCTCAGCAGTTTAC ***(NotI)***	Full length- *C. obscurus* (5' end)
LT61	**GCGGCCGC** TTAGTTGCGCTTTTTGCCATAG ***(NotI)***	Full length- *C. obscurus* (3' end)

Degenerate nucleotide **Y:** C or T; **R:** A or G; **H:** A, T or C; **N:** A, C, G or T.

5.4 Results

5.4.1 Degenerate RT-PCR amplification of partial cDNA fragments of the putative Δ6 elongase genes

To clone putative elongase genes from *C. thromboides* and *C. obscurus* by using degenerate RT-PCR, several Δ6 elongases previously identified from fungi, algae and animals were aligned by the DNAstar MegAlign program. Two conserved regions, the only histidine rich motif (F-L-H-V-Y-H-H) and the tyrosine rich motif found in these elongases (Figure 5.1), were used to design two degenerate oligonucleotide primers for RT-PCR. Degenerate RT-PCR produced a ~150 bp fragment from RNAs of the two *Conidiobolus* species (Figure 5.2). The expected PCR products were gel purified and cloned into the TA cloning vector for sequencing. Only one distinct contig, homologous to polyunsaturated fatty acid elongases, was observed in *C. obscurus*, whereas five different contigs, one of which resembled elongases, were observed in *C. thromboides*.

Both putative Δ6 elongase gene fragments from *C. thromboides* and *C. obscurus* showed very high sequence similarity to a Δ6 elongase isolated from *Morteriella alpina* (≥89%). This incredibly high homology occurs in the short and highly conserved region where the degenerate primers were targeted. In reference to other elongase enzymes, the putative full-length elongase genes from *C. thromboides* and *C. obscurus* should be ~ 957 bp. Thus, it is expected that there still remained at least 546 bp at the 5' end and 273 bp at the 3' end of the genes to be retrived.

5.4.2 Rapid amplification of the cDNA ends (RACE) of the putative elongase genes

To retrieve the missing 5' and 3' ends of the elongase genes, various gene specific primers were designed from the partial cDNA fragments of the elongase genes for the RACE reactions (Table 5.1).

The first 5' RACE reactions were carried out with the initial reverse 5' RACE primers, LT19 and LT56, and the forward adapter primer (AP1), which generated faint and distorted bands, similar to the result of the initial 5' RACE of desaturase genes. To obtain

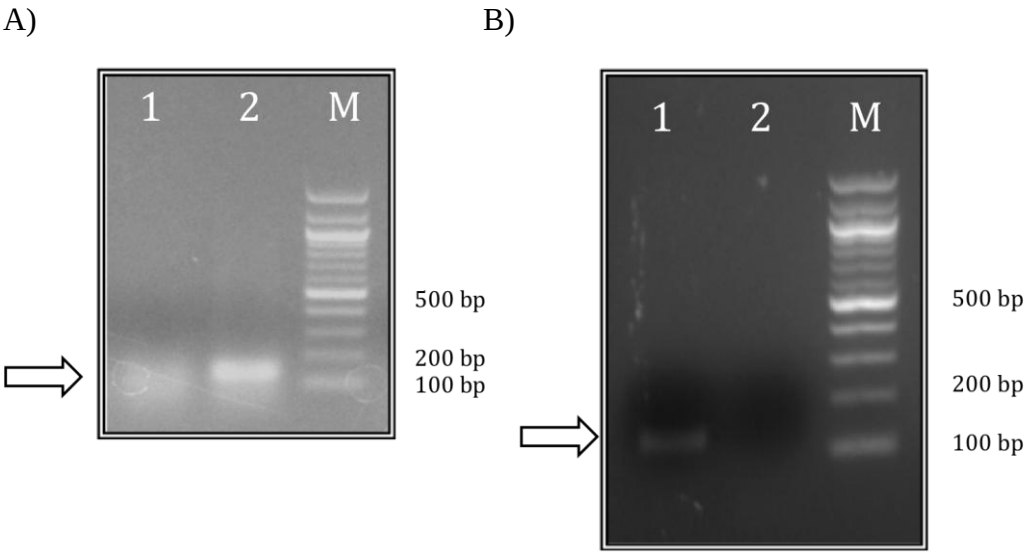


Figure 5.2: Degenerate RT-PCR of putative elongase genes. **A) *C. thromboides***: **1,** Negative control – LT5 and LT6 with no template; **2,** Sample – LT5 and LT6 with the RNA; **M,** 100 bp ladder marker. **B) *C. obscurus:* 1,** Negative control; **2,** Sample – LT5 and LT6 with the RNA; **M,** 100 bp ladder marker. **PCR Conditions:** 94°C for 1 minute, 10 cycles of 94°C for 30 seconds, 50°C for 1 minute, 72°C for 1 minute, 30 cycles of 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute and 72°C for 10 minutes.

the desirable RACE products, nested PCR reactions were performed using the initial RACE products as the templates and their respective nested reverse primers, LT32 and LT57, and the forward nested adapter primer (AP2), which produced the final RACE products with the expected size (Figure 5.3). These PCR products were gel purified and ligated into the TA cloning vector for sequencing analysis.

The sequencing results of the amplified fragments from the nested 5' RACE reactions from both fungal species indicated we have successfully retrived the missing 5' termini of both putative elongase genes. The amplified 5' fragment of the putative *CtE6* contains the start codon and an about 185 bp untranslated region, while the amplified 5' fragment of the putative *CoE6* contains the start codon and an about 163 bp untranslated region.

For the 3' RACE, the initial reactions with the cDNAs of *C. thromboides* and *C. obscurus* and their respective forward primers, LT18 and LT54, and the reverse adapter primer (AP1) also generated only faint bands, a result similar to that of the initial 5' RACE. Therefore, the nested 3' RACE reactions were performed using the initial 3' RACE PCR products as templates. Both nested reactions of *C. thromboides* and *C. obscurus* produced observable bands (Figure 5.4).

The nested PCR products were then cut and gel purified and cloned into the TA cloning vector for sequencing analysis. The sequencing results indicated, both the 3' termini of the elongase genes from *C. thromboides* and *C. obscurus* were successfully isolated. The amplified 3' fragment of the putative *CtE6* contains a termination codon (TAA) and about 97 bp of an untranslated region. The amplified 3' fragment of the putative *CoE6* contains a termination codon (TAA) and a 94 bp untranslated region.

5.4.3 Cloning the full-length elongase genes from *C. thromboides* and *C. obscurus*

With the successful retrieval of both the 5' and 3' ends of putative elongase genes, the full-length genes could then be assembled using the RACE products and the central cDNA fragments generated by degenerate RT-PCR. Sequence assembly revealed that the full-length cDNA of the putative Δ6 elongase from *C. thromboides* (*CtE6*) was 1272 bp

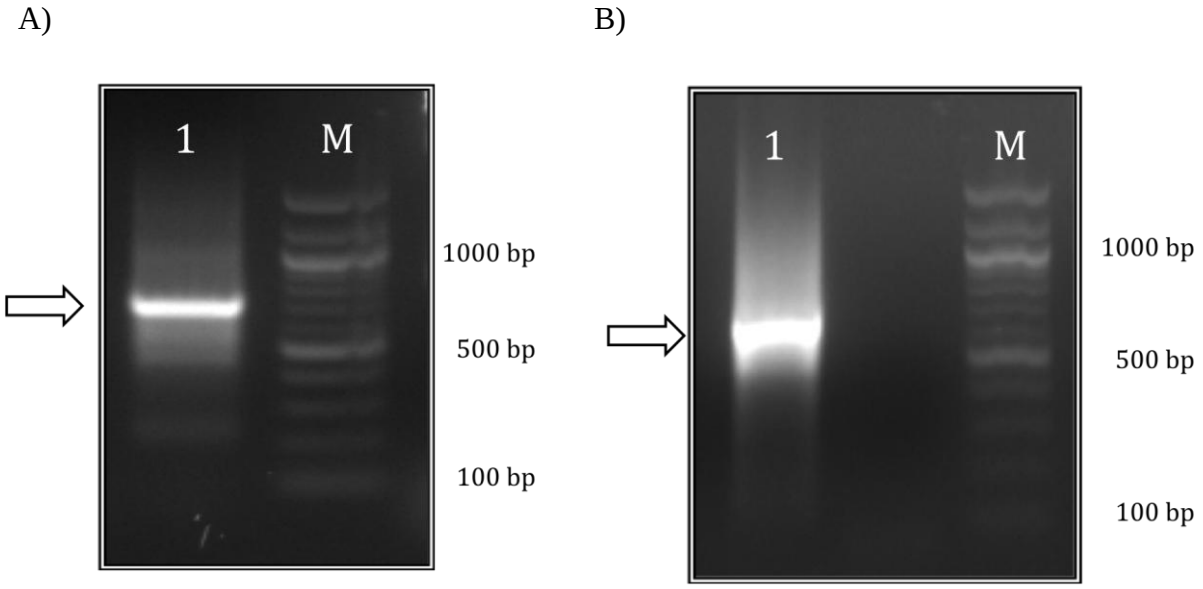
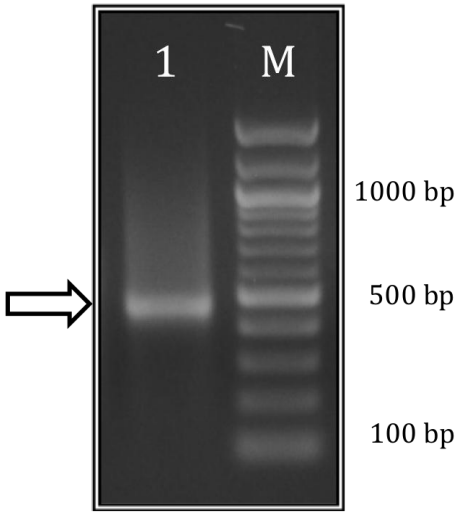


Figure 5.3 The nested 5' RACE reaction for retieving the 5' ends of elongase genes. **A),** ***C. thromboides*****: 1,** the nested reaction with LT32 and AP2; **M,** 100 bp ladder marker. **PCR conditions**: 94**°**C for 2 minutes, 35 cycles of 94°C for 30 seconds, 68°C for 2 minutes. **B)** ***C. obscurus*****: 1**, the nested reaction with LT57 and AP2; **M,** 1000 bp marker. **PCR conditions**: 94**°**C for 2 minutes, 35 cycles of 94°C for 30 seconds, 68°C for 30 seconds, 72°C for 1 minute.

A)



B)

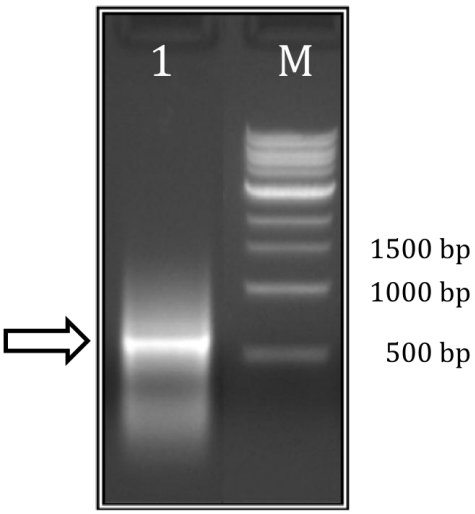


Figure 5.4 Results of the nested 3' RACE for retrieving the 3' ends of elongase genes. **A),** ***C. thromboides*****: 1,** the nested PCR reaction with primers LT20 and AP2; **M,** 100 bp ladder marker. **PCR conditions**: 94**°**C for 2 minutes, 35 cycles of 94°C for 30 seconds, 68°C for 2 minutes. **B)** ***C. obscurus*****: 1,** the nested PCR reaction with primers LT55 and AP2; **M,** 1000 bp ladder marker. **PCR conditions:** 94**°**C for 2 minutes, 35 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 2 minutes.

in length, while the full-length cDNA from *C. obscurus* (*CoE6*) was 1241 bp long. The putative start codons of *CtE6* and *CoE6* were identified by the presence of in-frame stop codons upstream of the initiation site. The upstream stop codon of *CtE6* was found just adjacent to the start site, while the upsteam stop codon of *CoE6* was found at 60 bp upstream from the start site. In both cases the putative start site was confirmed through multiple sequence alignments. Having determined the start and stop codons, the two ORFs of Δ6 elongase cDNAs from *C. thromboides* and *C. obscurus* were amplified by RT-PCR using gene-specific primers which correspond to the nucleotide sequences between the start and stop codons of the ORFs. To facilitate the subsequent manipulation in functional studies, the 5' ends of both forward and reverse primers were added with restriction sites (Table 5.1). The PCR products from *C. thromboides* and *C. obscurus* were gel purified and cloned into pYES2.1/V5-His-TOPO for sequence analysis.

5.4.4 Sequence analysis of the putative Δ6 elongases from *Conidiobolus spp.*

The sequencing results of the full-length *CtE6* cDNA retrieved by specific RT-PCR indicated there was only one cDNA species of the putative Δ6 elongase gene from *C. thromboides*. The *CtE6* gene contains an ORF of 990 bp with a start codon located at 185 bp from the 5' end, and the stop codon located at 97 bp from the 3' end. The ORF of *CtE6* encodes a polypeptide of 330 amino acids with a molecular weight of 38.1 kDa (Figure 5.5).

Sequencing results of the full-length *CoE6* cDNA retrieved by specific RT-PCR also indicated the presence of only one cDNA species for the putative Δ6 elongase gene. The *CoE6* gene encodes an ORF of 984 bp with a start codon at 163 bp from the 5' end and a stop codon at 94 bp from the 3' end. The ORF of *CoE6* encodes a polypeptide of 328 amino acids with a molecular weight of 37.3 kDa.

```
TAGGCAAGGTGTAACGTCAGTTATGATGGCGGTGTGCAGCAAGTGATACAACGAGAACGGTAGAAAGATGTTCAAGTTGG

TACCCAGGTTTCCATAGTGAGACAAGTACACCCGCTGTTGTTGTCAGGCAGGAGATATTACAAATTGGACCTAAGAGTTG

GTCTAGTGCGTCTAAGGGGTCCATCCGTGGGTCGCATACCGAAGACGGAGAGTAAATAATATCCGTACATAACAGCATGA

ATAATGAGTTTATTAAATACATTGGATACTATTACTTCAAGCAATAATGTTGTATCAGCATACAACGATGCCCCAGTAG
  M  S  L  L  N  T  L  D  T  I  T  S  S  N  N  V  V  S  A  Y  N  D  A  P  V

ACTATTTAATTAAAGTAGTAGATTTAGCTTTAACTGCTAACAAAGCAGTCTTCAATGTTATAGAAGCCAAAGTTAACGTA
D  Y  L  I  K  V  V  D  L  A  L  T  A  N  K  A  V  F  N  V  I  E  A  K  V  N  V

TGGATGCCAACATTGATGATAAACTTAAGAGAACAGGTCTCTAATTTAATCTCACCAATAAGTAAATACTTGCCATTGTT
 W  M  P  T  L  M  I  N  L  R  E  Q  V  S  N  L  I  S  P  I  S  K  Y  L  P  L  L

AGATCCTATCGAAGTGTTTTCTATCTTGTTTTTATATATCTTTGTTGTGTTTTTTTGGCTCAAAGTAGCTTCTAGCTTCC
  D  P  I  E  V  F  S  I  L  F  L  Y  I  F  V  V  F  F  W  L  K  V  A  S  S  F

TCCCACGTTTCGAAGTAAGATTATTTTCCCTTTTCCATAATTTCTGTATGGTCGTTTTATCCGCCTATATGTGCTCTTCC
L  P  R  F  E  V  R  L  F  S  L  F  H  N  F  C  M  V  V  L  S  A  Y  M  C  S  S

ATCCTATTACAAGCTTATGCAGATAAGTATATTCTATTCACTAACCCCGTCGATCACTCTCCAAATGGTATTCCAATGGC
 I  L  L  Q  A  Y  A  D  K  Y  I  L  F  T  N  P  V  D  H  S  P  N  G  I  P  M  A

TAAAATAATATGGTTATTTTATATTTCCAAAATCCCAGAGTTCGTTGACACTATGATCATGTTGGTTAAACAAAACTACC
  K  I  I  W  L  F  Y  I  S  K  I  P  E  F  V  D  T  M  I  M  L  V  K  Q  N  Y

GCCAAATCTCCTTYTTACAYGTCTACCAYCATAGTTCGATCTTTGCTATTTGGTGGATTGTTACCTTGATGGCACCAAAT
R  Q  I  S  F  L  H  V  Y  H  H  S  S  I  F  A  I  W  W  I  V  T  L  M  A  P  N

GGTGATGCTTATTTCTCAGCTGCATTGAACTCATTTATTCATGTTGTTATGTACGGMTAYTAYTTMCTCTCTGCACTTGG
 G  D  A  Y  F  S  A  A  L  N  S  F  I  H  V  V  M  Y  G  Y  Y  L  L  S  A  L  G

ATTCAAATCCGTCTCCTTTGTTAAGAAATATATTACTATGGGACAAATGACTCAATTTGCACTCAACTTTGTTCAAGCTA
  F  K  S  V  S  F  V  K  K  Y  I  T  M  G  Q  M  T  Q  F  A  L  N  F  V  Q  A

GTTATAATATTGTAGACAGAAATTACTTACGTCCACAAGTCCATGAGCAAGGATTAGCCTATCCTTATGCTCTTTCCGTT
S  Y  N  I  V  D  R  N  Y  L  R  P  Q  V  H  E  Q  G  L  A  Y  P  Y  A  L  S  V

TTACTTTGGTTCTATATGATCTCTATGTTGGTGTTATTCGCTAACTTTTATATTCAAGATCGTATCCGTCAATCAAAGTT
 L  L  W  F  Y  M  I  S  M  L  V  L  F  A  N  F  Y  I  Q  D  R  I  R  Q  S  K  L

AAAGTCTCAACAAAAGGGAAAGAAAATGAATTAGTATATAAAATGTTATTGTTATAAATAAAATAAAAAATTTAATTGAA
  K  S  Q  Q  K  G  K  K  M  N  .

AAAAAAAAAAAAAAAAAAAAAAAAAGCGGCCGCTGAATTCTAACCTGCCCGG
```

Figure 5.5 The full-length cDNA of *CtE6* and the translated ORF. The conserved histidine and tyrosine boxes of the elongases are highlighted. The stop codon found in the 5' upsteam is indicated by an arrow.

Figure 5.6 The full-length cDNA sequence of *CoE6* and the translated ORF. The conserved histidine and tyrosine boxes of the elongases are highlighted. The stop codon found in the 5' upsteam is indicated by an arrow.

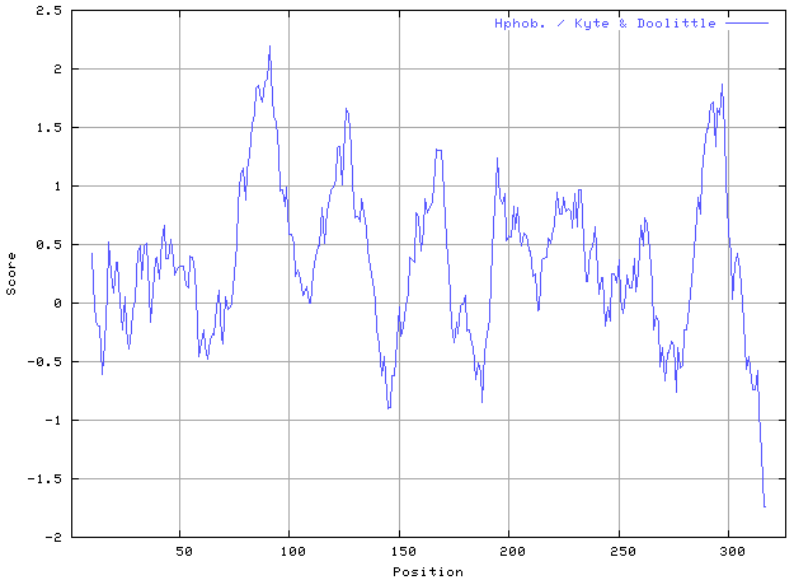
Hydrophobic plot analysis of these two putative Δ6 elongase polypeptides revealed that both possess two highly hydrophobic domains. One is located at about 60 amino acids downstream the N-terminus, while the other resides at the C-terminus. Similar hydropathy profiles were also observed in other *Δ*6 elongases from the green microalga *Parietochloris incisa* (Iskandarov *et al*., 2008) and fungus *Mortierella alpina* (Parker-Barnes *et al*., 2000).

The two deduced Δ6 elongase polypeptides CtE6 and CoE6 share 55% of amino acid identity throughtout the sequences. Comparison of these two sequences with other known Δ6 elongases showed that *CoE6* has high amino acid identity to elongases from *Mortierella alpina* (52%), *Physcomitrella patens* (41%), *Ostreococcus tauri* (40%), *Marchantia polymorpha* (36%), and *Thraustochytrium sp.* (39%), while CtE6 has high amino acid sequence identity with the elongases from *Mortierella alpina* (53%), *Marchantia polymorpha* (42%), *Thraustochytrium sp.* (42%), *Ostreococcus tauri* (40%) and *Physcomitrella patens* (39%). These results suggested that the two cDNAs *CtE6* and *CoE6* might encode functional Δ6 elongases involved in the synthesis of VLCPUFAs such as arachidonic acid and eicosapentaenoic acid in *C. thromboides* and *C. obscurus*.

The alignment of CtE6 and CoE6 with other polyunsaturated fatty acid (PUFA) elongase enzymes revealed both contain four conserved motifs (KxxE/DxxDT, the extended histidine box QxxFLHxYHH, the tyrosine box NxxxHxxMYxYY and TxxQxxQ) that are typical for this group of elongases (Figure 5.8). The homology among elongase sequences from different organisms mainly occurs in these highly conserved regions (Leonard *et al*., 2003).

Phylogenetic analysis of CoE6 and CtE6 with related elongases, as shown in Figure 5.9, indicated that these elongases were clustered into three distinct groups: 1) 18C/20C PUFA elongases from chordates that can elongate more than one type of polyunsaturatedfatty acids (PUFAs), including XiELO, an elongase from *Xenopus laevis* able to elongate polyunsaturated fatty acids with various chain length (Meyer *et al*,. 2004); 2) Saturate (SFA)/mono-unsaturate (MUFAs) elongases from fungi and vertebrate

A)



B)

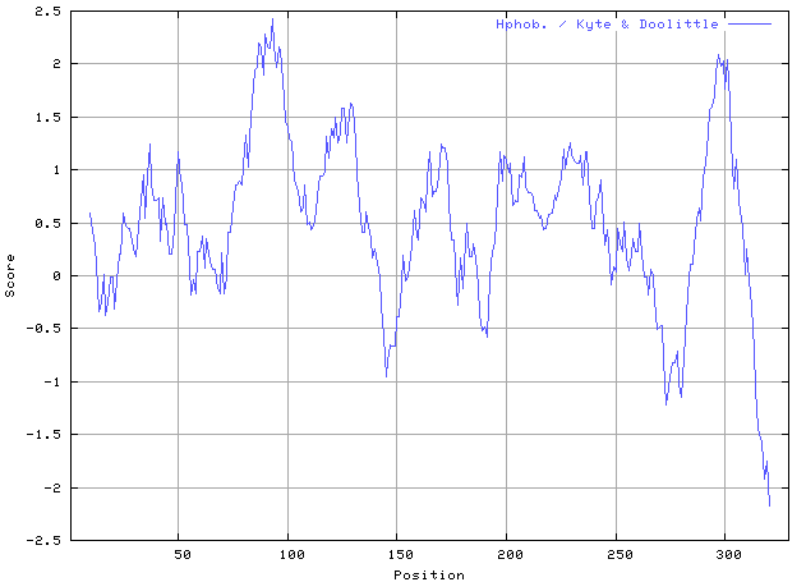


Figure 5.7 Kyte-Doolittle hydropathy plots of CoE6 (A) and CtE6 (B). Protscale was used to determine the hydrophobic profile of the polypeptide based on Kyte & Doolittle parameters (Kyte and Doolittle, 1982). Window size, 19. Peaks greater than 1.6 in vertical axis indicate the presence of a hydrophobic domain.

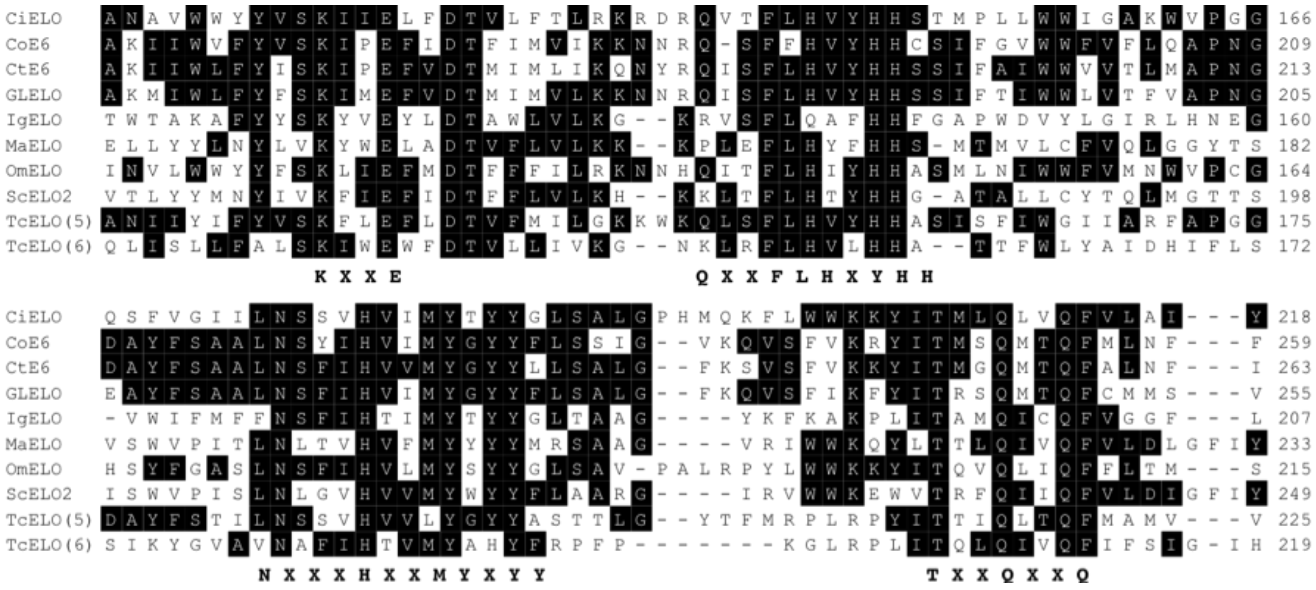


Figure 5.8 Clustal alignment of elongase enzymes from *Conidiobolus obscurus* (**CoE6**), *Conidiobolus thromboides* (**CtE6**), *Mortierella alpina* (**GLELO**, AAF70417), *Thraustochytrium aureum* (**TcELO5** CAM55873, **TcELO6,** CAJ30819), *Isochyrsis galbana* (**IgELO**, AAL37626), *Saccharomyces cerevisiae* (**ScELO2**, NP_009963), *Ciona intestinalis* (**CiELO**, NP_001029014), *Oncorhynchus mykiss* (**OmELO,** AAV67803). Shaded in the dark black are the regions of the protein sequences that are shown to be highly conserved throughout the species.

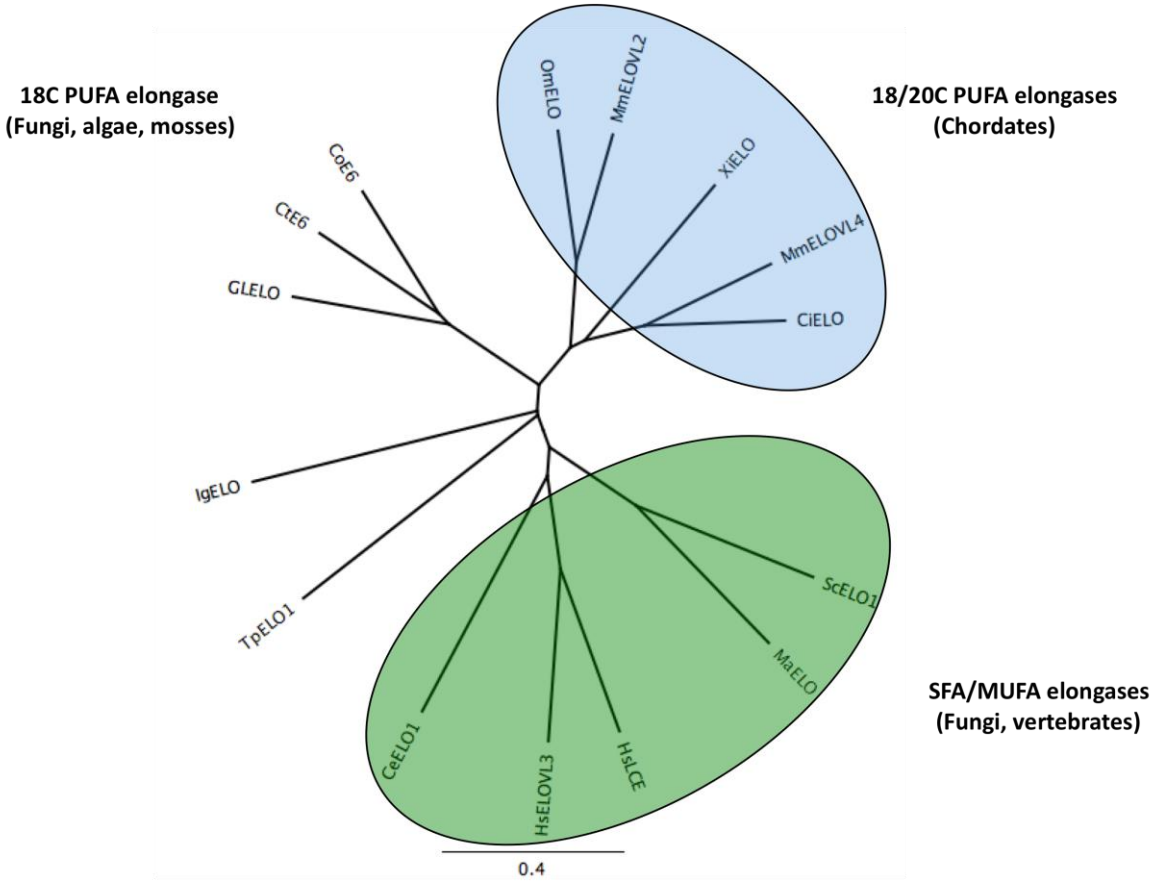


Figure 5.9 Phylogenetic analysis of **CoE6** and **CtE6** with related sequences. The DDBJ/EMBL/GenBank accession numbers of the different protein sequences are as follows: *Mortierella alpina* (**MaELO,** AAF71789), *S. cerevisiae* (**ScELO1,** NP_012339), *C. elegans* (**CeELO1** AAF70462), M. *musculus* (**MmELOV2,** NP_062296), M. *musculus* (**MmELOV4,** AAG47667), *Mortierella alpina* (**GLELO,** AAF70417), *Physcomitrella patens* (**PpELO,** AAL84174), *Isochrysis galbana* (**IgELO,** AAL37626), Ciona intestinalis (**CiELO,** NM_001033842), *Homo sapiens* (**HsELOVL3,** NM_152310), *Homo sapiens* (**HsLCE,** NP_076995), *Oncorhynchus mykiss* (**OmELO,** NP_001118108), *Thalassiosira pseudonana* (**TpELO1,** AAV67800), and *Xenopus laevis* (**XiELO,** NP_001082390). The branch comprising five 18C PUFA elongases was not marked to show its primary delineation. The unrooted phylogram was constructed by the UPGMA method using Geneious 4.70.

that can elongate saturated and mono-unsaturated fatty acids, but not any PUFAs, including yeast ScELO1; and 3) 18C PUFA elongases from lower organisms that can only elongate 18C Δ6 desaturated PUFAs, including the two elongases cloned from *Conidiobolus spp.* (CtE6 and CoE6).

5.4.5 Functional analysis of putative Δ 6 elongases from *C. thromboides* and *C. obscurus*

To determine the funciton of *CtE6*, the ORF was cloned into a yeast expression vector pYES 2.1 under the guidance of the *GAL1* promoter, which was then transformed into *S. cerevisiae* (INVSc1). The transformants were selected on –URA selective media and screened by PCR using a primer in the promoter and a primer in the ORF of *CtE6*.

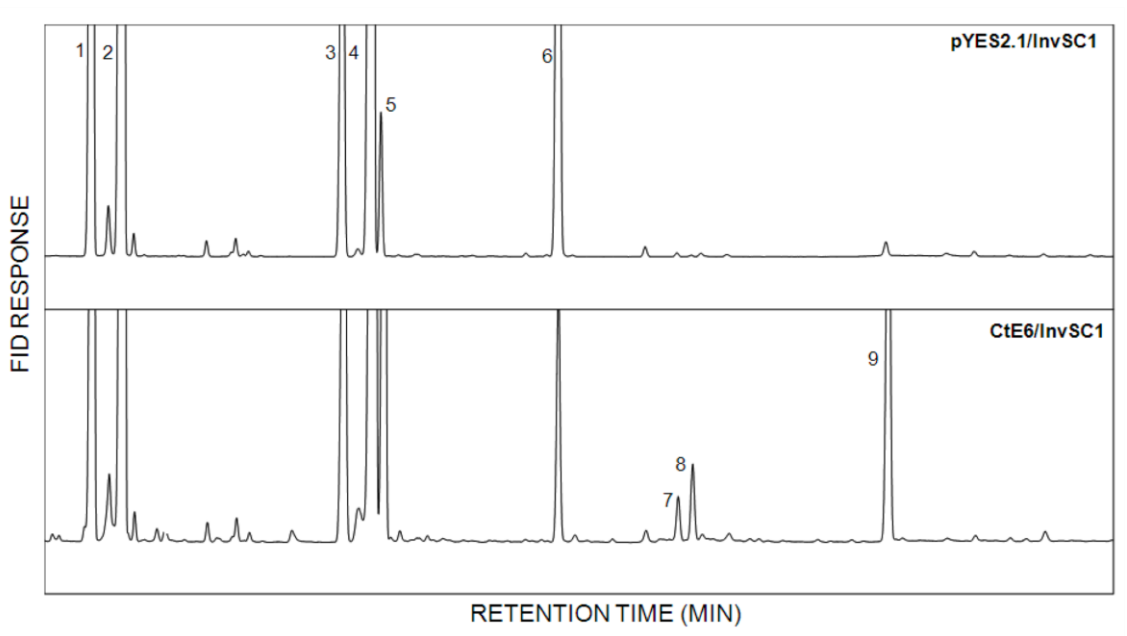
The selected transformants were grown in the presence of γ-linolenic acid or stearidonic acid, two substrates for the Δ6 elongase. Fatty acid analysis indicated that in comparison with the control, the transformants expressing *CtE6* in the presence of GLA produced a new peak with the retention time identical to that of DGLA. GC/MS analysis of this peak confirmed it is indeed 20:3-8,11,14, DGLA, indicating *CtE6* isolated from *C. thromboides* encoded a functional elongase which is responsible for the addition of a two carbon unit to the carboxyl end of the Δ6 desaturated fatty acid (Figure 5.10).

Similarily, to define the function of *CoE6*, the ORF was cloned into the yeast expression vector under the control of GAL promoter. The plasmid was transformed into *S. cerevisiae* INVSc1. Fatty acid analysis showed that compared with the control, transformants expressing *CoE6* in presence of γ-linolenic acid produced a new peak with the same retention time as dihomo-γ-linolenic acid. GC/MS analysis of this peak confirmed it is 20:3-8,11,14, DGLA, indicating that *CoE6*, similar to *CtE6*, encoded functional elongase that could elongate γ-linolenic acid.

To study the substrate specificity of CoE6 and CtE6, the transformants were supplied with several other fatty acids that differed in the number and position of the double bonds as well as in the chain length. The results of the feeding experiments showed that the highest elongation activities of either enzyme was detected on 18:3-6,9,12 (GLA) and 18:4-6,9,12,15 (SDA). However, a difference in the activities was noticed between the two enzymes, which could be

used to distinguish one from another. CoE6 was observed to effectively elongate both GLA and SDA with the high conversion efficieny (50% and 59%, respectively). Besides, CoE6 could also, at a reduced efficiency, elongate 17:1-10 and 18:3-9,12,15 producing 19:1-12 and 20:2-11,14, 17, respectively. The longer chain fatty acids (>18) were not substrates of CoE6 (Table 5.2). On the other hand, CtE6 was also observed to effectively elongate GLA and SDA; however, in comparison to *CoE6*, the conversion effiency of CtE6 was almost ten percent greater, reaching 67% and 70% on GLA and SDA, respectively. In addition, *CtE6* was also found to elongate other substrates effectively, such as 17:1-10, 18:2-9,12 and 18:3-9,12,15 with their elongation effeciencies reaching more than 20%. Furthermore, CtE6 was capable of elongating the VLPUFAs, such as 20:4-5,8,11,14 (ARA) and 20:5-5,8,11,14,17 (EPA), albeit at a lower effeciency (~10%) (Table 5.3). It is noteworthy that although the phylogenetic analysis showed that CoE6 and CtE6 were classified as 18C PUFA elongases, both possessed activites towards multiple types of polyunsaturated fatty acids. In particular, CtE6 has elongating activity to both 18C Δ6 and 20C Δ5 desaturated fatty acids.

A)



B)

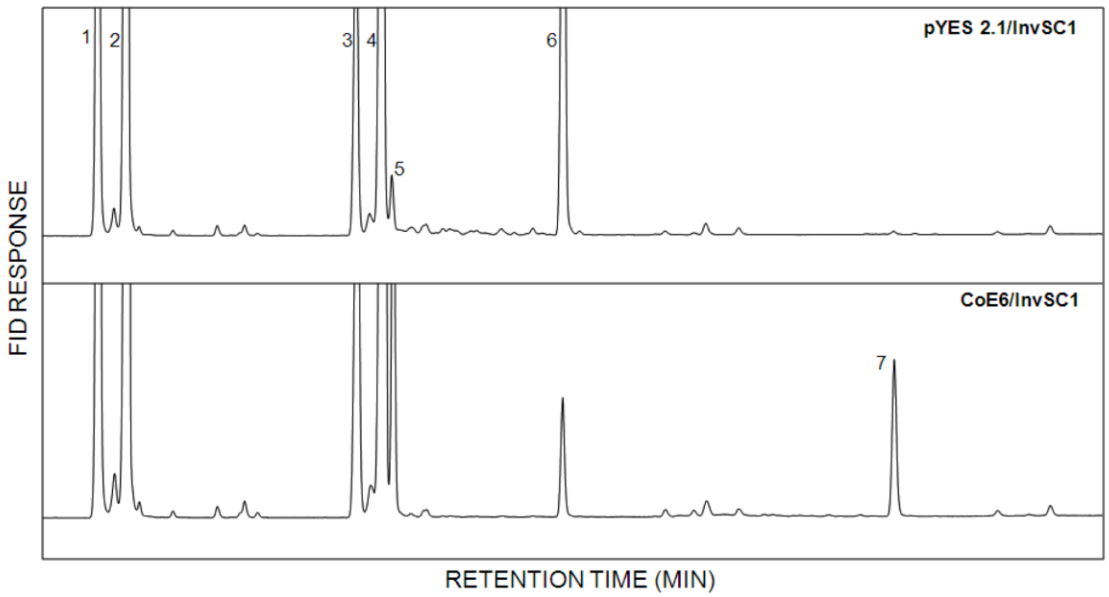


Figure 5.10 Functional analysis of *CtE6* (A) and *CoE6* (B) with stearidonic acid (18:4-6,9,12,15). **Fatty acid peaks in (A):** (1) 16:0, (2) 16:1-9, (3) 18:0, (4) 18:1-9, (5) 18:1-11, (6) 18:4-6,9,12,15, (7) 20:1-11, (8) 20:1-13, (9) 20:4-8,11,14,17. **Fatty acid peaks in (B):** (1) 16:0, (2) 16:1-9, (3) 18:0, (4) 18:1-9, (5) 18:1-11, (6) 18:4-6,9,12,15, (7) 20:4-8,11,14,17.

Table 5.2 The conversion efficiency of the yeast strain *CtE6*/INVSc1 expressing *C. thromboides* Δ6 elongase with exo/endogenous substrates

Substrate		Product		Conversion Efficiency
Fatty Acid	**% TFA**	**Fatty Acid**	**% TFA**	
17:1-10	5.23±0.07	19:1-12	3.09±0.57	36.94±3.88
18:1-9	28.07±0.40	20:1-11	0.47±0.01	2.06±0.02
18:1-11	11.66±0.11	20:1-13	0.82±0.02	6.56±0.12
18:2-9,12	5.73±0.12	20:2-11,14	2.19±0.12	27.68±0.76
18:3-6,9,12	1.58±0.06	20:3-8,11,14	3.27±0.15	67.45±1.84
18:3-9,12,15	8.59± 0.14	20:3-11,14,17	3.51±0.18	22.48±0.81
18:4-6,9,12,15	1.95±0.14	20:4-8,11,14,17	4.37±0.22	69.96±0.54
20:4-5,8,11,14	5.22±0.14	22:3-7,10,13,16	0.41±0.02	7.79±0.45
20:5-5,8,11,14,17	3.56±0.13	22:5-7,10,13,16,19	0.51±0.03	12.59±0.28

Note: Values represented as the wight percentage of total fatty acids (TFA%).

Table 5.3 The conversion efficiency of the yeast strain *CoE6*/INVSc1 expressing *C. obscurus* Δ6 elongase with exogenous substrates

Substrate		Product		Conversion Efficiency
Fatty Acid	**% TFA**	**Fatty Acid**	**% TFA**	
17:1-10	7.25±2.93	19:1-12	0.39±0.14	5.10±0.28
18:3-6,9,12	4.72±1.81	20:3-8,11,14	4.65±1.61	49.87±3.03
18:3-9,12,15	11.26±2.04	20:3-11,14,17	1.98±0.25	15.04±1.56
18:4-6,9,12,15	1.43±0.11	20:4-8,11,14,17	2.10±0.04	59.50±1.36

Note: Values represented as the weight percentage of total fatty acids (TFA%).

5.5 Discussion

In this study, two cDNA sequences encoding for putative Δ6 elongase were isolated by degenerate RT-PCR and RACE from *C. obscurus* and *C. thromboides*. The full-length cDNA from *C. thromboides* (*CtE6*) contained an ORF of 990 bp and encoded a polypeptide of 330 amino acids, while the full length cDNA from *C. obscurus* (*CoE6*) contained an ORF of 984 bp and coded for a polypeptide of 328 amino acids. Phylogenetic analysis of CoE6 and CtE6 with related sequences indicated that the two putative elongases isolated from *Conidibolus* species were clustered into the same clade representing elongases specific for the elongation of 18C Δ6 desaturated fatty acids. This clade of elongases were distantly separated from two other clades, one clade comprised elongases specific for elongation of saturated and mono-unsaturted fatty acids and the other consisted of elongases for elongation of both 18C and 20C PUFAs. Although both CoE6 and CtE6 shared high sequence similarity (67%) and were clustered into the same clade of elongases, they possessed quite different enzymatic properties. CoE6 elongated only 18C PUFAs, as do others in the group, while CtE6 was able to elongate a broad range of fatty acids ranging from 18C to 20C fatty acids with one to five double bonds such as oleic acid, linoleic acid, and linolenic acid, GLA, SDA, ARA and EPA. Elongation activity of CtE6 on VLCPUFAs such as ARA and EPA was not anticipated as it was clustered into the 18C PUFA elongase. In addition, *C. thromboides* where the gene was isolated does not produce any VLCPUFAs-elongated products. Thus, it would be of interest to know the structural determinants of the two-elongase enzymes responsible for distinct substrate preference. Nevertheless, it was worth noting that CtE6 has a wider range of substrates, the most preferred substrate was Δ6 desaturated products such as GLA and SDA. In fact, in comparison to other similar enzymes, CtE6 appeared to have very high activity on GLA and SDA. The conversion efficiencies on the two substrates accounted for 67% and 69%, respectively.

6.0 Study 4: Reconstitution of the ETA pathway in *Saccharomyces cerevisiae*

6.1 Abstract

To reconstitute ETA biosynthesis pathways, *CoD6* - a functional Δ6 desaturase gene and *CoE6* - a functional Δ6 elongase gene from *C. obscurus* were first co-expressed in yeast. Transformants expressing the two genes produced DGLA and ETA when exogenously supplied with linoleic acid and alpha-linolenic acid, respectively. Co-expressing of the four genes, *CoD6, CoE6, CpDes12* - a Δ12 desaturase gene and *CpDesX* - a ω3 desaturase gene from *Claviceps purpurea*, achieved the reconstitution of the entire ETA biosynthetic pathway. Production of ETA was accomplished without feeding of any exogenous substrate. The level of ETA in transformants expressing the four genes reached 0.1 % of the total fatty acids.

6.2 Hypothesis

Co-expressing all genes involved in the biosynthesis of VLCPUFAs in yeast would allow us to examine how these genes work together to produce DGLA and ETA, which could provide the guidance for metabolic engineering of these two nutraceutical fatty acids in plants.Reconstituion of the ETA pathway in *Saccharomyces cerevisiae* using the functional *CoD6, CoE6, CpDes12* - a Δ12 desaturase gene and *CpDesX* - a ω3 desaturase gene from *Claviceps purpurea* would produce final expected product, ETA.

6.3 Experimental approach

6.3.1 Construction of yeast co-expression plasmids containing *CoD6* and *CoE6*

The yeast co-expression vector pESC-HIS (Stratagene, La Jolla, California) (Figure 6.1) was used to clone *CoD6* and *CoE6* where the desaturase gene was under the regulation of *GAL1* promoter and the elongase gene was under the control of *GAL10* promoter. *CoD6* was released from pLT11 with BamHI restriction enzyme, while *CoE6* was released from pLT12 with NotI restriction enzyme. Each gene was sequentially ligated into its respective site within the pESC-HIS vector. The two-gene plasmid was then transformed into *E. coli* TOP10 F' electro-competent cells. The transformant cells were plated on ampicillin selective media and the transformants were screened through colony PCR using a gene specific primer (LT59 or LT61)

in combination with a vector primer (*GAL1* or *GAL10*), respectively. Once the correct recombinant plasmid (pLT14) was identified, it was then transformed into the yeast strain INVSc1 and the yeast transformants were selected on a histidine deficient medium.

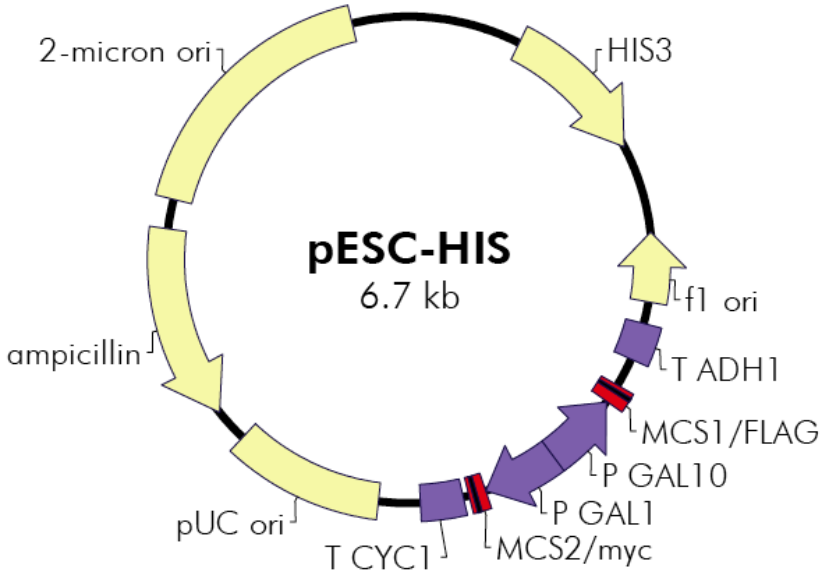
6.3.2 Construction of yeast co-expression plasmids containing *Claviceps purpurea* Δ12 desaturase (*CpDes12*) and ω-3 desaturase (*CpDesX*) genes

The yeast co-expression vector pESC-URA (Stratagene) (Figure 6.1) was used to clone *CpDes12* and *CpDesX* where *CpDes12* was under the control of *GAL1* promoter and *CpDesX* was under the control of the *GAL10* promoter. *CpDesX* and *CpDes12* were kindly provided by Dr. Meesapuodsuk, University of Saskatchewan. To facilitate the *CpDesX* cloning process, Bgl II restriction site was incorporated at the 5' ends of the forward and reverse primers (LT48 **GAAGATCTTC**GAAATGGCTAACAAATCTCC and LT49 **GAAGATCTTC**CTAGCCGTGTGTGTGGAC). These primers were then used to amplify the full-length *CpDesX*, the amplified product was digested with Bgl II and inserted into its respective digested site within the pESC-URA vector. For cloning *CpDes12*, the plasmid containing the gene was cut with the restriction enzymes BamHI and EcoRI, and ligated into the sites of pESC-URA. The recombinant plasmid was selected on ampicillin media and screened by PCR using a gene specific primer (LT48 and LT49) in combination with the respective a vector primer (*GAL1* and *GAL10*). The correct recombinant vector containing the two genes was designated as pLT10. To express four genes in yeast, the *S. cerevisiae* INVSc1 was transformed with pLT10 and pLT14, simultaneously. The yeast transformants were selected on -HIS and -URA media.

6.3.3 Fatty acid analysis of yeast transformants

To assess CoD6 and CoE6 co-expression, the transgenic yeast cells were grown to saturation in 10 mL of synthetic yeast media lacking histidine and containing 2% glucose, and 0.67% bacto-yeast nitrogen base for 2 days at 28°C. The cultures were then washed twice with distilled water and induced with 10 mL of the induction medium (the synthetic

A)



B)

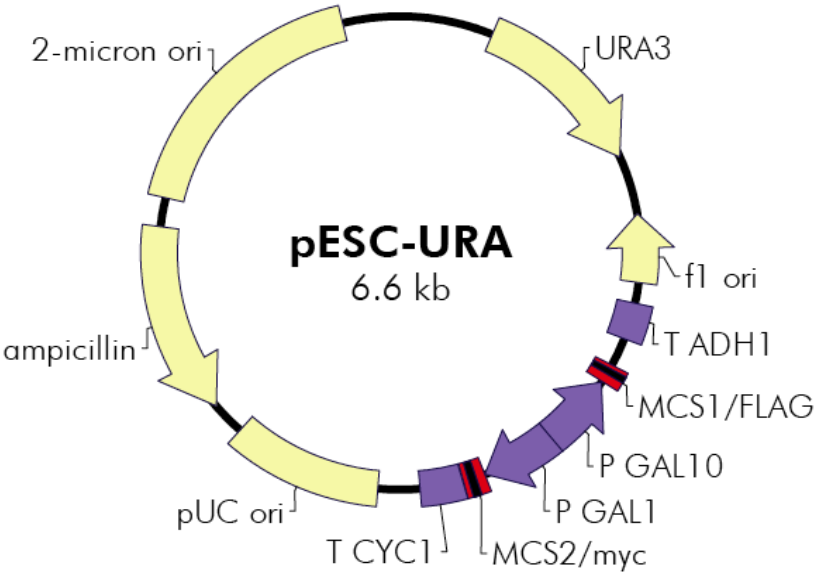


Figure 6.1 The pESC yeast expression systems were used to reconstruct the VLCPUFA biosynthetic pathway. pESC-HIS was used to co-express CoD6 and CoE6 (A), while pESC-URA was used to co-express CpDesX and CpDes12 (B).

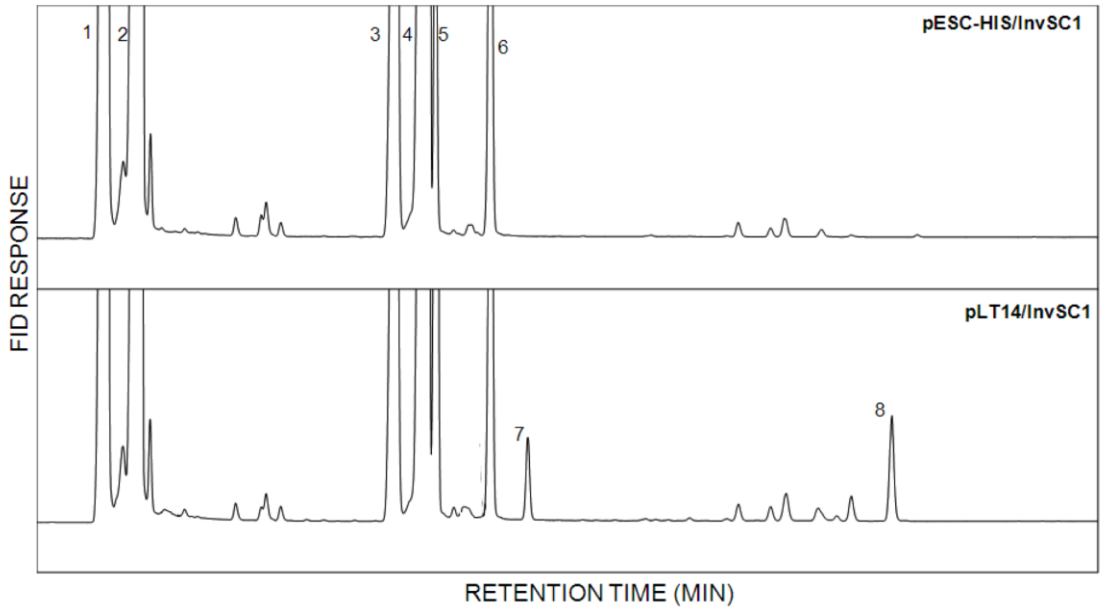
yeast medium containing 2% galactose) supplemented with or without 500 μM fatty acid substrate in the presence of 0.1% tergitol. The induced cultures were incubated at 20°C for 4 days shaking at 250 rpm to allow for adequate gene expression and accumulation of VLCPUFAs. Following the induction, the yeast cells were harvested by centrifugation at 2400 rpm and washed once with 15 mL of 0.1% tergitol and twice with 10 mL of distilled water. The total fatty acid methyl esters (FAMEs) were prepared directly from washed yeast pellets with 3N methanolic HCl. The FAMEs samples were analyzed on an Agilent 6890N gas chromatograph equipped with a DB-23 column (30 m) with 0.25-μm-film thickness (J&W Scientific). The column temperature was maintained at 160 °C for 1 min, then raised to 240 °C at a rate of 4 °C/min.

6.4 Results

6.4.1 Co-expression of CoD6 and CoE6 in *S. cerevisiae*

The first approach for reconstitution of the ETA pathway in yeast was to co-express *CoD6* and *CoE6*, the two functional genes from *C. obscurus*. *CoD6* was put under the guidance of *GAL1* promoter, while *CoE6* was put under the guidance of the *GAL10* promoter in pESC-URA. The two gene plasmid was then transformed into yeast *S. cerevisiae* (INVSc1). Transformants were selected on a -HIS selective medium and screening by PCR. When fed with linoleic acid (LA) or α linolenic acid (ALA), two precursors for the synthesis of DGLA and ETA, respectively, the control yeast harboring the empty vector did not produce any new fatty acids. However, yeast transformants expressing the two genes, when exogenously supplied with LA, produced two major new fatty acids which were identified by GC/GC-MS as 18:3-6,9,12 (GLA) and 20:3-8,11,14 (DGLA), and when exogenously supplied with ALA, produced three major new fatty acids which were identified by GC and GC-MS as 18:4-6,9,12,15 (SDA), the Δ6 desaturated product of 20:3-11,14,17 (ALA), the elongated products of ALA and 20:4-8,11,14,17 (ETA), the elongated product of SDA (Figure 6.2). These data indicated that in presence of LA or ALA, the transformants could produce DGLA in the ω6 pathway or ETA in the ω3 pathway.

A)



B)

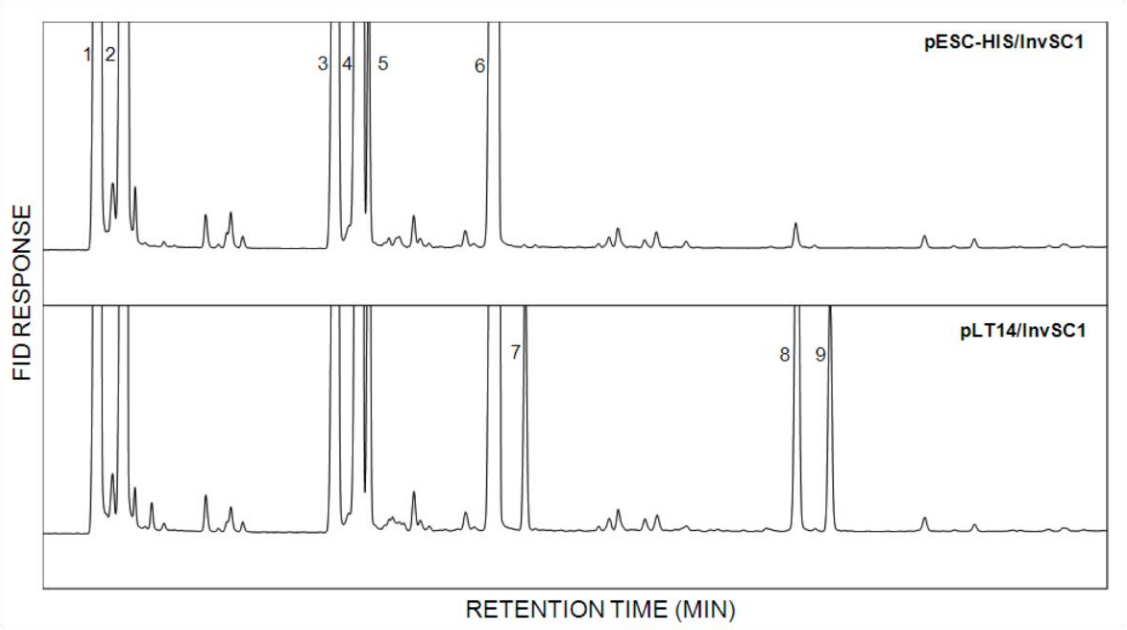


Figure 6.2 Co-expression of two genes *CoD6/CoE6* in yeast with exogenously supplied linoleic acid (A) and α-linolenic acid (B). **(A) Fatty acid peaks:** (1) 16:0, (2) 16:1-9, (3) 18:0, (4) 18:1-9, (5) 18:1-11, (6) 18:2-9,12, (7) 18:3-6,9,12 and (8) 20:3-8,11,14. **(B) Fatty acid peaks:** (1) 16:0, (2) 16:1-9, (3) 18:0, (4) 18:1-9, (5) 18:1-11, (6) 18:3-9,12,15, (7) 18:4-6,9,12,15, (8) 20:3-11,14,17 and (9) 20:4-8,11,14,17.

6.4.2 Reconstitution of the entire ETA pathway in *S. cerevisiae*

The entire ETA pathways was reconstituted by expressing four genes, *CoD6, CoE6, CpDesX*, an ω3 desaturase gene that can introduce a ω3 double bond in LA, and *CpDes12*, a desaturase that can introduce a Δ12 double bond in oleic acid. *CpDesX* was put under the guidance of the *GALI* promoter, while *CpDes12* was put under the guidance of the *GAL10* promoter in pESC-HIS. The recombinant plasmid was then co-transformed with pESC-URA-CoD6-CoE6 into yeast *S. cerevisiae* (INVSc1). Transformants were selected on a –HIS/–URA selective medium and identified by PCR.

Yeast lacks both Δ12-desaturase and ω3 desaturase enzymes, thus is unable to produce α-linolenic acid (18:3-9,12,15), a precursor for Δ6 desaturation and subsequently Δ6 elongation for the ETA biosynthesis. However, yeast naturally produce substantial amounts of oleic acid (18:1-9), a precursor of LA and ALA synthesis catalyzed by Δ12-desaturase and ω3 desaturase. Therefore, *S. cerevisiae* transformants expressing the four genes was expected to be able to synthesize ETA without exogenous supplementation of any fatty acids (Figure 6.4).

The fatty acid analysis showed that compared with the control, transformants expressing four genes *CoD6, CoE6, CpDesX and CpDes12* produced ten novel peaks with the retention times and mass spectra identical to those of the Δ12 and ω3 desaturated products 18:2-9,12 (LA) and 18:3-9,12,15 (ALA), Δ6 desaturated products 18:3-6,9,12 (GLA) and 18:4-6,9,12,15 (SDA), and Δ6 elongated products 20:3-8,11,14 (DGLA) and 20:4-8,11,14,17(ETA), as well as 16:2-9,12, the Δ12 desaturated product of 16:1-9, 16:3-9,12,15, the Δ15 desaturated product of 16:2-9,12, 18:2-11,14, the elongated product of 16:2-9,12 and 20:3-11,14,17, the elongated product of 18:3-9,12,15 (Figure 6.3), four side products of the reconstituted pathways (Figure 6.4). This result indicated that the entire DGLA and ETA pathways were successfully reconstituted in yeast. The reconstitution of the DGLA pathway in yeast *S. cerevisiae* was previously reported (Yazawa *et al*., 2007), but there is no report so far describing the reconstitution of the entire ω3 ETA pathway in yeast. However, it was noted that although the Δ6 desaturation/elongation ω3 pathway was reconstituted in this study, the level of ETA was still low (~ 0.1%) (Table 6.1). The rate limiting reaction in the pathway appeared to be the Δ6 desaturase step where less than

7% of the substrates were converted into corresponding Δ6 desaturated products. The low Δ6 desaturation might be due to low activity of *CoD6* in yeast.

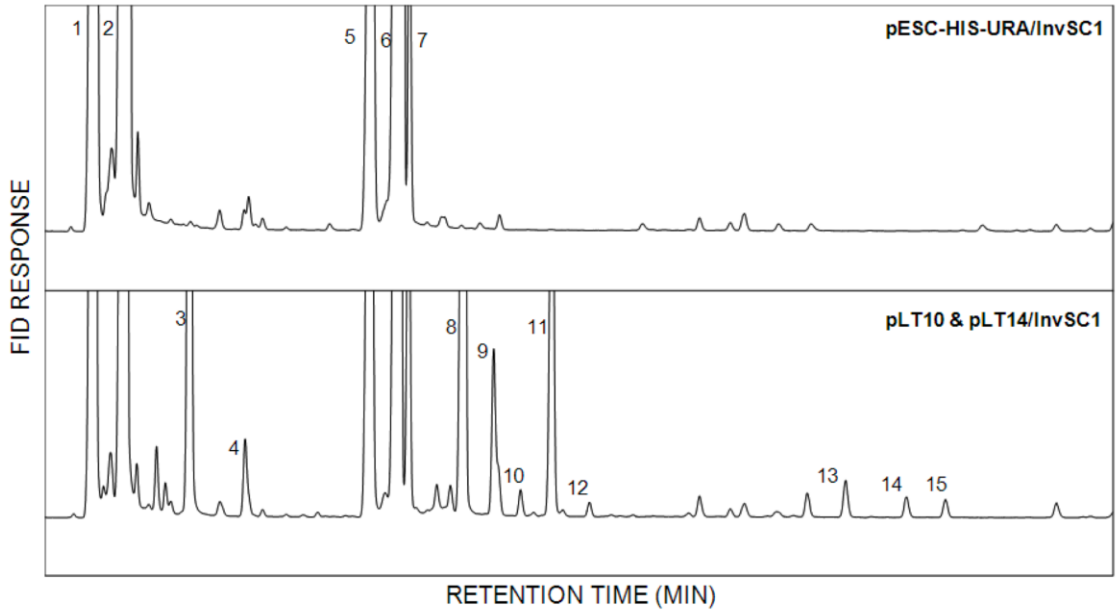


Figure 6.3 Reconstitution of the entire ETA pathway in yeast by co-expressing four genes *CD6/CoD6E/CpDes12/CpDesX*. **(A) Fatty acid peaks:** (1) 16:0, (2) 16:1-9, (3) 16:2-9,12, (4) 16:3-9,12,15, (5) 18:0, (6) 18:1-9, (7) 18:1-11, (8) 18:2-9,12, (9) 18:2-11,14, (10) 18:3-6,9,12, (11) 18:3-9,12,15, (12) 18:4-6,9,12,15, (13) 20:3-8,11,14, (14) 20:3-11,14,17 and (15) 20:4-8,11,14,17.

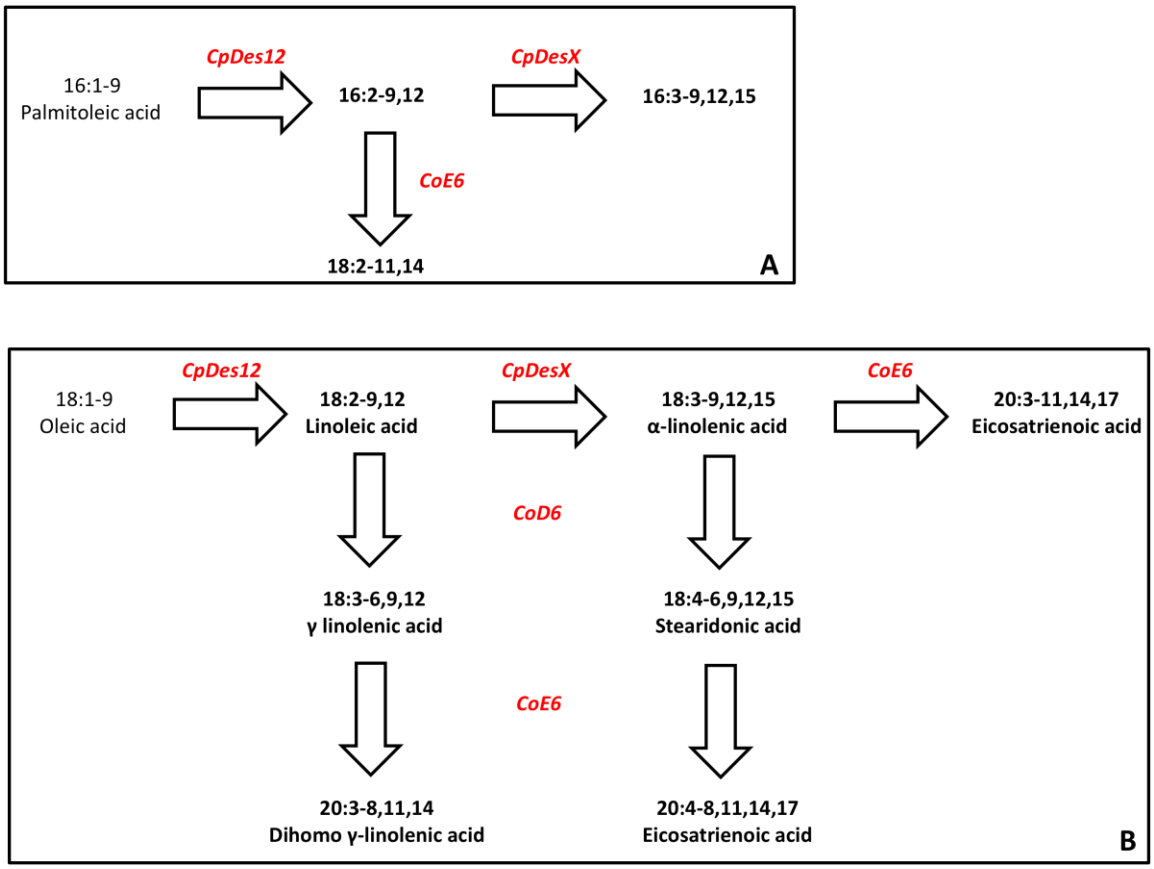


Figure 6.4 A diagram illustrating the VLCPUFA biosynthetic pathway of the yeast transformants containing the four genes, *CpDes12*, *CpDesX*, *CoE6,* and *CoD6*. A), 16 carbon metabolic pathways and B), 18 – 20 carbon metabolic pathways. Indicated in bold are the novel products produced by the yeast transformants.

Table 6.1 Fatty acid composition of the yeast transformants containing the 4 gene construct (weight percent of the total fatty acids, %TFA).

Fatty Acids	**%TFA**	**Conversion efficiency (%)**	**Fatty Acids**	**%TFA**	**Conversion Efficiency (%)**
16:0	19.08±0.28		**18:2-11,14**	1.03±0.02	8.21±0.10
16:1-9	32.99±0.58		**18:3-6,9,12**	0.13±0.01	4.25±0.13
16:2-9,12	6.99±0.24	15.36±0.25	**18:3-9,12,15**	2.79±0.08	27.97±1.18
16:3-9,12,15	4.50±0.42	35.95±0.69	**18:4-6,9,12,15**	0.09±0.01	6.30±0.31
18:0	7.06±0.26		**20:3-8,11,14**	0.21±0.01	3.97±0.99
18:1-9	18.05±0.32		**20:3-11,14,17**	0.12±0.01	62.97±0.92
18:2-9,12	7.67±0.42	37.63±1.21	**20:4-8,11,14,17**	0.11±0.01	55.31±5.41

6.5 Discussion

To reconstruct the ETA pathway in yeast, we initially used two genes *CtD6* and *CtE6* cloned from the same fungal species *C. obscurus* that encoded a functional Δ6 desaturase and a functional Δ6 elongase, respectively. The result from the study above showed that Δ6 elongase CtE6 from *C. thromboides* exhibited a higher elongation activity on Δ6 desaturated products compared to CoE6, however, it has a wider array of substrates from 18C to 20C fatty acids with varying double bonds. On the contrary, CoE6 had a much narrower range of substrates, and preferentially only elongated the 18C Δ6 desaturated substrates. Thus, it was assumed that using this enzyme with a narrower substrate preference to reconstitute the pathway would increase the production of the final product by eliminating or minimizing the side products that might arise from the non-specific elongation. With the exogenous supply of linolenic acid, transformants expressing *CoD6* and *CoE6* genes indeed produced the final ETA product that accounted for approximately 1% of the total fatty acids in the cells.

After the successful reconstruction of the partial ETA pathway with the two genes, the reconstitution of the entire ETA pathway was then undertaken by co-expressing four genes simultaneously, *CoD6, CoE6, CpDes12* - a Δ12 desaturase gene and *CpDesX* a ω3 desaturase gene from *C. purpurea*. Transformants expressing the four genes produced ten new fatty acids including the final product ETA without any supplementation of exogenous substrates.

Although the entire ETA pathway was successfully reconstituted in yeast, the level of the final product ETA is still low, accounting for about 0.1 % of the total fatty acids. Many factors could affect the yield of the final product in a reconstituted metabolic pathway, which might include the activity of the transgene *per se*, the choice of the host expression system, the activity of a promoter used to control the transgene, and growing environment of tranformants. Yazawa *et al.* demonstrated that use of constitutive promoters controlling the expression of transgenes and alteration of medium composition especially the content of nitrogen could increase the production of the final product in the reconstituted DGLA pathway in yeast (Yazawa *et al*., 2007).

The calculation of conversion efficiencies on each step of the reconstituted VLCPUFA pathway in the transformants revealed that the bottleneck of the reconstituted pathway was the Δ6 desaturation where the conversion efficiency was only about 6%, far from that of the Δ6 elongation step (50%). Part of the reason for the low Δ6 desaturation could be attributed to the relatively low activity of CoD6 as shown previously. In addition, comparison of conversion efficiencies between the single-gene functional study and the multi-gene reconstitution showed CoE6 elongase in the reconstituted pathway was able to keep similar elongation efficiency (50-60%) as in the functional study, whereas CoD6 was unable to do the same, and the desaturation efficiency was reduced to about 6% in the reconstituted pathway from 15% in the functional study. The exact reason why the Δ6-desaturation efficiency was much lower in the multi-gene reconstitution than that in the single-gene functional expression was unknown. It has been demonstrated by previous studies that the major obstacle in the reconstitution of the VLCPUFA pathway in heterologous systems such as yeast and plant is the substrate availability, as desaturation and elongation involved in the biosynthesis of VLCPUFAs often occur in different lipid pools. It is generally believed that the elongase usually uses acyl-CoA as substrate, while most desaturases use glycerolipids as substrates (Lopez Alonso *et al*., 2003). However, a few acyl-CoA desaturases that can uses acyl-CoA as substrate have recently been identified in microalgae (Domergue *et al*., 2005). If the desaturases and elongase in the metabolic pathway do not use the same pool of substrates, the VLCPUFA biosynthesis would be less efficient, as it has to involve constant shuffling of the acyl intermediates between different pools (Abbadi *et al*., 2004; Domergue *et al*., 2003). At the moment, we do not have any information regarding the substrate form of CpDesX and CoD6 used in the ETA reconstitution. If CpDesX does not share the same form of substrates as CoD6, subsequent Δ6 desaturation by CoD6 could be affected by ineffective channeling of the substrate.

Utilizing pathway-specific enzymes could also help effectively reconstitute a VLCPUFA pathway. Many genes in the biosynthesis of VLCPUFAs have been identified from a variety of living organisms, but almost all of them use both ω3 and ω6 substrates. However, a Δ6 desaturase was recently cloned from *Primula vialli* (PvD6), which showed a strong preference towards ω3 linolenic acid (Sayanova *et al*., 2006). If this kind of pathway-specific enzyme is used to reconstitute the ω3 VLCPUFA pathway, the yield of the final ω3 product could be

higher, as undesirable non-specific intermediates in the ω6 pathway would be eliminated or minimized.

7.0 General summary and conclusions

Very long chain polyunsaturated fatty acids (VLCPUFAs) are very important in maintaining normal functions of the human body. Dietary supplementations of VLCPUFAs have been shown to provide protection against cardiovascular diseases and to enhance performance of eyes, brains and nerve systems. Currently, the main source of these fatty acids is marine fish and fish oil. However, with the rapid dwindling of the fish population in oceans, alternative sources of these fatty acids are needed to meet the growing demand for these fatty acids.

Metabolic engineering of oilseed crops has been considered as one of the most cost-effective and sustainable alternatives for VLCPUFAs. Thus, the main goal of this project was to identify and characterize novel Δ6 desaturase and Δ6 elongase genes from entomopathogenic fungi which have been previously shown to accumulate VLCPUFAs, but from which genes involved in the biosynthesis of these fatty acids have yet to be identified. Ultimately, it was hoped that the genes encoding novel Δ6 desaturase and Δ6 elongase from these fungi could be used for metabolic engineering of plants to produce VLCPUFAs as dietary supplements.

This thesis research first started with fatty acid analysis of five different entomopathogenic fungal species. Those species that produced high amounts of VLCPUFAs would be selected as gene sources for Δ6 desaturase and Δ6 elongase involved in the biosynthesis of DGLA (dihomo-γ-linolenic acid, 20:3-8,11,14) and ETA (eicosatetraenoic acid, 20:4-8,11,14,18), precursors for the biosynthesis of ARA and EPA. Fatty acid analysis showed *Conidibolous obscurus* produced a high level of ARA (arachidonic acid, 20:4-5,8,11,14) and EPA (eicosapentaenoic acid, 20:5-5,8,11,14,17), while *Conidibolous thromboides* produced a high level of ARA only. Thus, these two species producing the greater amount of Δ6 desaturated and elongated products were selected for further gene cloning studies.

To clone the Δ6 desaturase gene from *Conidiobolous spp.*, degenerate RT-PCR and RACE strategies were adopted. The degenerate primers were designed to target several highly conserved regions of Δ6 desaturases previously identified from other species, including one

heme-binding site and three histidine boxes. By using degenerate RT-PCR, partial cDNA fragments of desaturase genes from *C. obscurus* and *C. thromboides* were obtained. The missing 5' and 3' ends of the genes were retrieved through 5' and 3' RACE methods. To determine the function of the putative desaturase genes, the two full-length cDNAs were separately expressed in the yeast *S. cerevisiae* INVSc1. Expression studies indicated CoD6 was a functional Δ6 desaturase enzyme with substrate preference towards linoleic acid and α-linolenic acid with the similar conversion efficiency, whereas CtD6 exhibited no desaturase activity when expressed in yeast.

Similarly, degenerate RT-PCR and RACE were used to clone Δ6 elongases from the two *Conidibolus* species. The degenerate primers were designed to target the two highly conserved regions, the histidine box and the tyrosine box, of the elongases previously identified from other organisms. The degenerate RT-PCR generated partial cDNA fragments of the elongase genes from *C. obscurus* and *C. thromboides*. The 5'and 3' RACE retrieved the missing 5' and 3' ends of the two putative elongase genes. Expression studies in yeast indicated that both full-length cDNAs encoded functional Δ6 elongase enzymes. CtE6 elongated a wide array of substrates such as Δ9 fatty acids LA and ALA, Δ6 fatty acids DLA and SDA, and Δ5 fatty acids ARA and EPA with the highest elongation efficiency on the two Δ6 fatty acids reaching 67% and 70%, respectively, whereas CoE6 mainly elongated GLA (50%) and SDA (59%).

With successful identification of Δ6 desaturase and Δ6 elongase from the fungal species, we then undertook the task of reconstituting the entire ETA biosynthetic pathways in yeast. This was done by co-expressing four genes simultaneously, *CoD6* encoding a Δ6 desaturase from *C. obscurus*, *CoE6* coding for a Δ6 elongase from *C. obscurus*, *CpDesX* for a ω3 desaturase from *C. purpurea* and *CpDes12* for a Δ12 desaturase from *C. purpurea*. Without exogenous fatty acid supplementation, the transformants expressing the four genes used the endogenous substrate to synthesize ETA. The level ETA in transgenic yeast reached 0.1% of the total fatty acids.

Although the ETA biosynthetic pathways were successfully reconstituted in yeast, the level of final products was still low. The poor Δ6 desaturation step was identified as the bottleneck of the reconstituted pathway. Although the exact reason of the low Δ6 desaturation in the reconstituted

pathways is unknown, we believe that it might be due to the low activity of the cloned Δ6 desaturase from *C. obscurus* and/or the substrate availability for this enzyme in the reconstituted pathway. Future direction, therefore, will be how to improve the Δ6 desaturation efficiency, thereby increasing the ETA production. For instance, rationally designed site-directed mutagenesis can be used to increase the Δ6 desaturase activity. Elongases and desaturases with the same substrate form may be used for reconstituting the pathway to improve the substrate availability and enhance intermediate flux, thereby increasing the final products in the metabolic pathways.

8.0 References

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